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THE PROTEINASE INACTIVATION OF CATALASE AND SOME PROPERTIES OF THE DIGESTS*

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(Received for publication, November 25, 1957)

In a previous short-communication (*1*) the author reported that peptic digestion of beef liver catalase resulted in progressive inactivation of its catalatic activity and that the peroxidatic activity for pyrogallol appeared evidently. It has been of great interest in enzyme chemistry studies that hemoproteins are selectively constructed for their respective enzymic actions. Kaziro (*2*) has offered an opinion that all the hemoproteins possess, more or less, latent abilities of O_2- or H_2O_2-activation and of electron transport, of which some are substantially demonstrated by the specific configuration of the protein part, while the others are only revealed, for example, by attacking the protein moiety of the enzyme. In this connection Kaziro's collaborators (*3–8*) reported their investigations on mild modifications of hemoproteins, by using some kinds of perturbators, in relation to their catalytic actions.

In 1951 Tsou (*9*) isolated the pepsindigested cytochrome c, and demonstrated its ascorbic acid oxidase activity and autoxidizability. Later Tuppy and his coworkers (*10*, *11*) purified a ferriporphyrin c peptide from beef cytochrome c by peptic digestion, and they demonstrated its peroxidatic activity together with its amino acid sequence. Recently Kajita (*12*) presented a report on the cholehemopeptide prepared by proteinase digestion of verdohemoglobin.

In this paper the inactivation processes of catalase and a few properties of the pepsin- and trypsin-digested catalase are described.

EXPERIMENTAL

Materials

Crystalline Beef Liver Catalase—Catalase was precipitated from the aqueous extract of beef liver with acetone (*13*), and fractionated by ammonium sulfate (*14*). Catalase was further purified by recrystallization, three or four times (*15*). Crystalline catalase was then suspended in redistilled water, and dissolved at pH 7.2–7.4 by the addition of 2 *N*

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NaOH. The activity of this highly purified catalase solution was determined as Kat. f, 55,700 by the spectrophotometric method (*16*). This enzyme solution contained 254 mg. N and 1.3 mg. Fe per 100 ml. (average of two runs).

Crystalline Pepsin—Crystalline pepsin (Nutrition & Biochemical Co.), which was shown to have an activity of 0.2 Anson units per mg. N, was used.

Crystalline Trypsin—Crystalline pancreatic trypsin (Trypsillin) was kindly supplied from Mochida Co. Ltd. Trypsillin was shown to have an activity of 2.2 Anson units per mg. N.

Methods of Assay

Catalatic Activity—For determination of the catalatic activity (H_2O_2 decomposition) the modified procedure of Sizer (*16*) was applied to the digestion mixture. It consisted of measuring the extinction coefficient at 240 mμ per cm. (E_{240}) for 60 seconds at intervals, and the activity was calculated as the first order reaction: $K=(2.3/t(sec.))\log([H_2O_2]_0/[H_2O_2]_t)$.

Peroxidatic Activity—To a mixture of 2.0 ml. of 0.2 *M* acetate buffer (pH 5.0), 1.0 ml. of 1 per cent guaiacol and 1.0 ml. of 0.5 per cent H_2O_2, a 0.2 ml.-aliquot of the neutralized digestion mixture (see below) was mixed, and after standing for 30 minutes at room temperature, the reaction mixture was diluted to 10.0 ml. With the use of 0.2 ml. of distilled water instead of the sample, the blank was run. The extinction coefficient at 470 mμ per cm. (E_{470}) was read against the blank.

Indophenol Oxidase Activity—The Nadi reaction was run as follows: To a mixture of 2.0 ml. of *M*/15 phosphate buffer (pH 7.2), 0.5 ml. of 0.05 *M* alcoholic α-naphthol and 0.5 ml. of 0.05 *M* dimethyl-*p*-phenylenediamine, a 0.2 ml.-portion of the neutralized digestion mixture was added, left standing at room temperature for 5 minutes, and mixed with 3.2 ml. of 95 per cent alcohol. The extinction coefficient at 580 mμ per cm. (E_{580}) was read against the blank solution.

Rate of Proteolysis—With an aliquot of the digestion mixture the alcoholic HCl titration in acetone (*17*) was carried out.

Methods of Digestion

(a) In order to follow the proteolytic inactivation with time (*Expt. I*), 1 ml. of catalase solution (15 mg.) was adjusted to the desired pH value with HCl or NaOH, and diluted exactly to 2.0 ml. 1 ml.-aliquots of a pepsin solution in 0.01 *N* HCl (10 mg./ml.) and of a trypsin solution (10 mg./ml.) were adjusted to corresponding pH values, and diluted exactly to 2.0 ml. Catalase and proteinase solutions adjusted to corresponding pH were mixed, and incubated at 25° for 120 minutes. The proteinase digestion was run at pH 3.0–5.6 (pepsin) and at pH 5.0–9.6 (trypsin). The effect of the amount of proteinase was examined by using 15 mg. of catalase, 0–20 mg. of pepsin at pH 4.0 or 0–20 mg. of trypsin at pH 7.4.

(b) For determination of the peroxidatic activity and the rate of hydrolysis during inactivation of the catalase by peptic digestion (pH 4.0) or by tryptic digestion (pH 7.4) (*Expt. II*), 4 ml. of the catalase solution (30 mg.), which had been adjusted to pH 4.0 (peptic digestion) or pH 7.4 (tryptic digestion) and then diluted two-folds, were mixed with 4 ml. of a pepsin solution (20 mg.) at pH 4.0 or a trypsin solution (20 mg.) at pH 7.4, respectively. From the digestion mixture a 0.2 ml.-aliquot was pipetted out at intervals and mixed with 2.0 ml. of *M*/15 phosphate buffer (pH 8) for the peptic digest or 2.0 ml. of 0.1 *M* acetate buffer (pH 4.6) for the tryptic digest, to stop the proteolysis. These neutralized digests showed a pH value of about 7 (peptic digest) or about 5 (tryptic digest), and they were used for measuring the absorption spectra of the Soret band and the determination

of the peroxidatic activity. The alcoholic HCl titration was run with 0.2 ml.-portions of the digest pipetted out at intervals.

(c) *In Expt. III*, dealing with the properties of the peptic digest (pH 1.5), 34.5 ml. of the stock catalase solution (520 mg.) were adjusted to pH 1.5 with dilute HCl, to which 20 mg. of pepsin was dissolved (final volume, 55 ml.). For the tryptic digestion (pH 7.4), 34.5 ml. of the catalase solution (520 mg.) were adjusted to pH 7.4, and mixed with 10 ml. of a freshly dissolved trypsin solution in *M*/15 phosphate buffer (pH 7.4). The incubation was continued for 24 hours at 25°. The acid-treated catalase (the control for acid denaturation) was prepared from 10 ml. of the catalase solution (150 mg.) by adjusting to pH 1.5 with dilute HCl (final volume, 16.4 ml.), and the incubation (25°, 24 hours), successively.

Paper Electrophoresis—In order to test the components of the digests, paper electrophoresis was carried out with 0.05–0.1 ml. of the digests, the acid-treated catalase, and non-treated catalase. Electrophoresis was run in 0.1 *M* veronal buffer (pH 8.6; ionic strength, 0.1) at 150 v., 10 mA., for 5–6 hours. The filter paper was dried, stained with bromphenol blue, impregnated with paraffin, and the pattern was read by a densitometer (Natsume Co., Tokyo).

Solubility Test of the Digests in Ammonium Sulfate Solutions—To 10 stoppered centrifuge tubes each 5.0 ml. of ammonium sulfate solutions, varying in concentration at an interval of 10 per cent in saturation degree, were transferred, respectively. To a control tube, 5.0 ml. of distilled water were added. To each tube 0.2 ml.-aliquots of the digestion mixture (the peptic at pH 1.5, and the tryptic at pH 7.4 (*Expt. III*)) were mixed, kept in an ice-box overnight, and filtered. The extinction coefficients of these filtrates we e measured at 390 mμ (Soret band) and 275 mμ (protein peak).

Test for the Splitting of Hematin Group from the Digests—A portion of the peptic digest (pH 1.5) was carefully adjusted to pH 7.4 and diluted to twice its volume with *M*/150 phosphate buffer (pH 7.4). The acid-treated catalase solution was adjusted and diluted as above. The tryptic digest and non-treated catalase were merely diluted two fold with *M*/150 phosphate buffer (pH 7.4). From these solutions 0.2 ml.-portions of each were pipetted out, mixed with 5.0 ml. of acetone in stoppered centrifuge tubes. After standing for 30 minutes in the ice-water mixture, the acetone solutions were centrifuged, and the supernatants were examined spectrophotometrically at 390 mμ (the digests and the acid-treated catalase) or 405 mμ (non-treated catalase).

RESULTS

Experiment I

Proteolytic Inactivation of Catalase

Peptic Inactivation of Catalase in Weakly Acidic Solutions—The inactivation of catalase by acid alone was checked as shown in Fig. 1 (dotted lines) together with the peptic inactivation (solid lines). As expected from the optimum pH for peptic hydrolysis the peptic inactivation was observed at pH lower than 4.4, but prompt acid inactivation readily occurred at pH 3.0 or below. Since the peptic inactivation of catalase without significant acid inactivation was measurable at pH around 4, the process of the peptic inactivation was followed with various amounts of pepsin upto 20 mg. (Fig. 2). In such a weak acid solution a greater amount of pepsin was required for inactivating the catalatic activity.

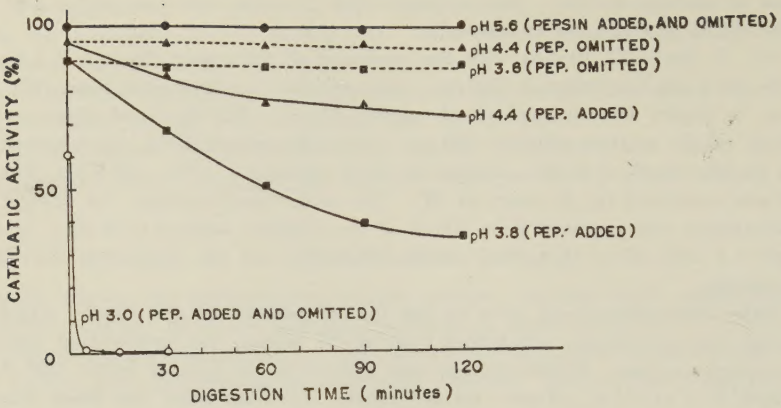


Fig. 1. Peptic inactivation of catalase.

The digestion mixture (4.0 ml.) contained 15 mg. of catalase and 10 mg. of pepsin. The catalatic activity (K_0' ($sec.^{-1}$, $mg.^{-1}$)) was evaluated by extrapolation graphically, and expressed as percentage of K_0' of non-treated catalase. For calculation of K value the amount of catalase (mg.) was calculated from the volune of the digestion mixture (15 mg. catalase/4.0 ml.) pipetted out for the determination of the activity, regardless of the peptic degradation.

Solid line ..... Peptic inactivation
Dotted line ..... Acid inactivation

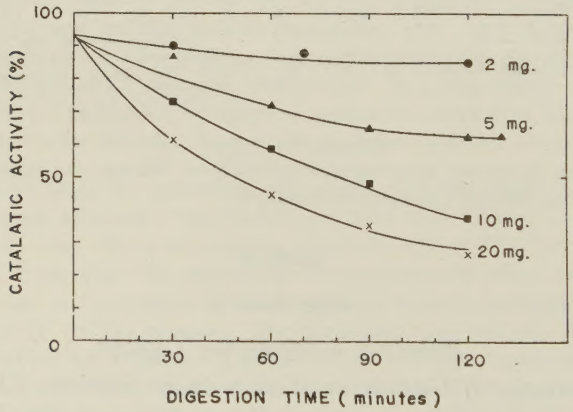


Fig. 2. The inactivation of catalase at pH 4.0 with pepsin varying in concentration.

The digestion mixture was 4.0 ml., and contained 15 mg. of catalase and 2, 5, 10, 20 mg. of pepsin, respectively as indicated in the figure.

Tryptic Inactivation of Catalase—The processes of catalase inactivation by trypsin at pH 5.0-9.6 are shown in Fig. 3 (solid lines). The rate of tryptic inactivation was greatest at pH 7–8, provided that the inactivation by alkali

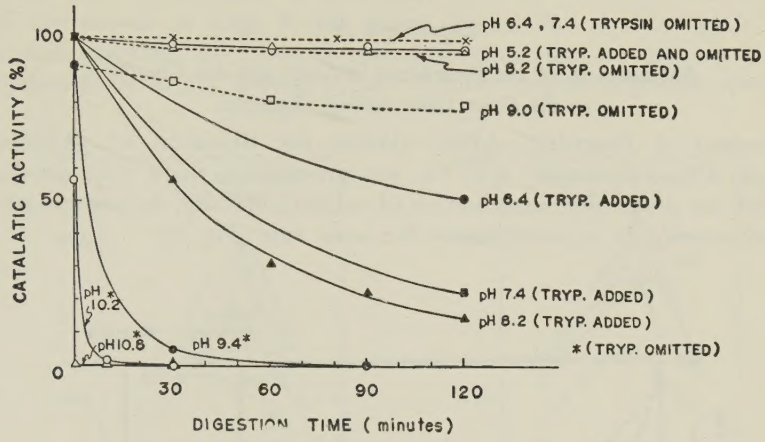


FIG. 3. Tryptic inactivation of catalase.

The digestion mixture was consisting of 15 mg. of catalase and 10 mg. of trypsin per 4 ml. Tryptic inactivation (solid line). Alkali inactivation (dotted line).

alone (dotted lines) should be avoided as in the present experiment. In *Expts. II* and *III*, therefore, the tryptic digestion was carried out at pH 7.4 to avoid any alkali inactivation. As no significant tryptic inactivation took place at pH about 5, the tryptic digestion in the experiments below was stopped by adjusting the digestion mixture to pH 5. The effect of the amount of trypsin (0–2 mg.) on the inactivation of catalase (1.5 mg.) was tested at pH 7.4 (Fig. 4).

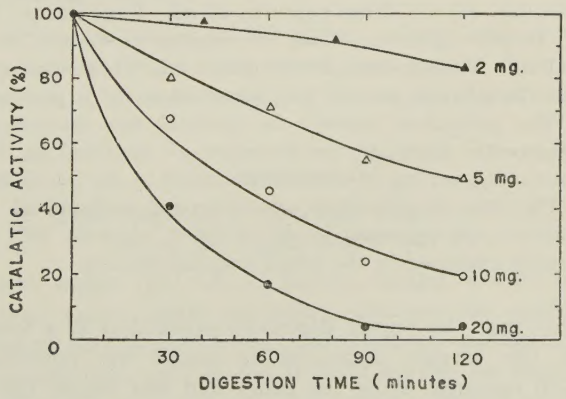


FIG. 4. The inaction of catalase at pH 7.4 by trypsin varying in concentration.

The digestion mixtures (4.0 ml.) contained 15 mg. of catalase and 2, 5, 10, and 20 mg. of trypsin, respectively.

Experiment II

Enhancement of the Peroxidatic Activity and the Change in the Soret Band during Digestion

Appearance of Peroxidatic Activity—While the digestion of catalase by pepsin (pH 4.0) or by trypsin (pH 7.4) was proceeding, there were produced, along with the progressive inactivation of catalatic activity, the peroxidatically active substance(s) at approximately the same rate (Fig. 5).

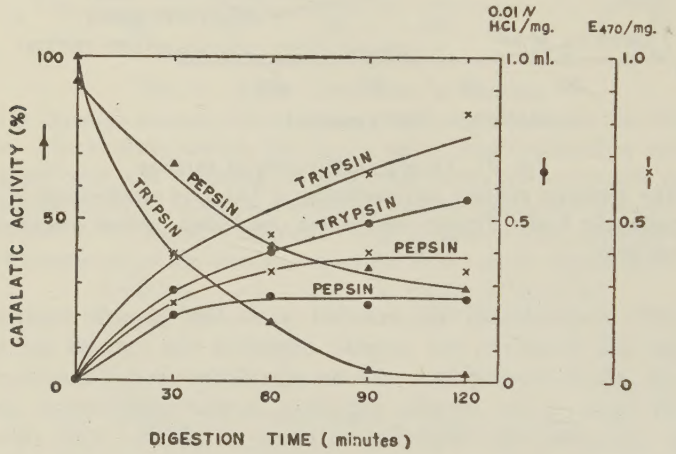


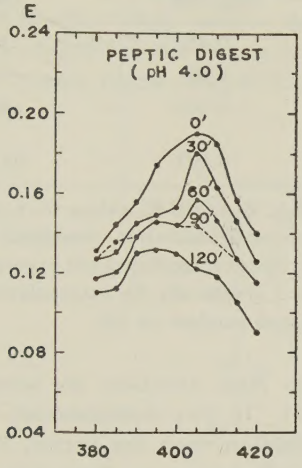
FIG. 5. The catalatic and peroxidatic activities during digestion of catalase by pepsin at pH 4.0 and trypsin at pH 7.4.

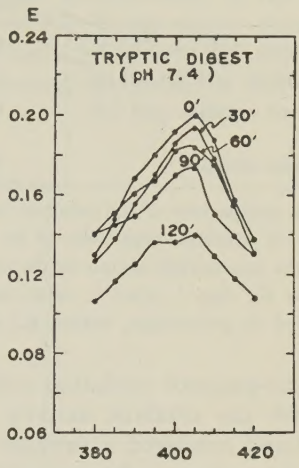
The reaction mixtures were prepared as follows: Peptic digestion: 30 mg. of catalase, 4.0 ml., 20 mg. pepsin in 4.0 ml. Final volume, 8.0 ml., pH at 4.0. Tryptic digestion: 30 mg. of catalase in 4.0 ml., 20 mg. of trypsin in 4.0 ml. Final volume, 8.0 ml., pH at 7.4. Incubation temperature at 25°. The catalatic activity was presented as K_0' in percentage, as in Fig. 1. The peroxidatic activity for guaiacol was determined with 0.2 ml. of neutralized digest, by the procedure as described in the text, and expressed as E_{470} per mg. of catalase contained in the initial digestion mixture. The rate of proteolysis was estimated with 0.5 ml. of the digestion mixture, and presented by ml. of 0.01 *N* alcoholic HCl titrated per mg. catalase contained in the initial reaction mixture.

It was noticed that the peptic digestion proceeded at a lower rate than the tryptic, and the former appeared to reach the equilibrium within approximately 120 minutes, while the latter did not reach the equilibrium within that time period.

The Change in the Soret Band—The absorption curve of the Soret band was measured with the neutralized digestion mixture. Since the digestion mixtures became, more or less, turbid when neutralized, the neutralized

sulutions were centrifuged, if necessary, prior to spectrophotometry. The results are shown in Figs. 6 (the peptic digest) and 7 (the tryptic digest). There was observed no isosbestic point, because the amount of the digest in supernatant was not always equal. But the peptic digestion resulted in





WAVE LENGHT (mμ)

FIG. 6. Soret band of the peptic digestion mixtures taken at interval of 30 minutes.

An aliquot of the digestion mixture, as described in Fig. 5, was mixed with 10 volumes of *M*/15 phosphate buffer at pH 7.2–7.4, and the supernatant by centrifugation, if any insoluble material was present, was used for spectrophotometry.

FIG. 7. Soret band of the tryptic digestion mixture taken at interval of 30 minutes.

The digestion mixture, as described in Fig. 5, was used by the similar way as in case of Fig. 6.

an increase in the absorption at 390–400 mμ along with a relative lowering of the absorption at 405 mμ (native catalase peak). Such a tendency was also demonstrated, to a lesser extent, in the tryptic digest during the course of digestion.

Experiment III

Some Properties of the Peptic Digest (pH 1.5) and of the Tryptic Digest (pH 7.4)

The peptic digest (pH 1.5, 24 hours) looked brown with a greenish tint and showed only a slight turbidity, whereas the acid-treated catalase (pH 15, 24 hours) was fairly turbid, had a brown precipitate and a diffuse dark green supernatant, and it was slightly soluble on neutralization.

Catalytic Properties of the Digests—The tryptic digest was shown to possess a trace of catalatic activity (0.4 per cent of the staring catalase), whereas a prompt acid inactivation had already occurred in the peptic digest (pH 1.5) as well as in the acid-treated catalase (pH 1.5). The catalytic activities for

TABLE I

The Catalytic Activities of the Digests

	Peroxidatic activity (E_{470}/mg.)	Oxidatic activity (E_{580}/mg.)	Catalatic activity (K_0')
Peptic digest (pH 1.5, 24 hrs.)	0.78	0.15	0.0%
Tryptic digest (pH 7.4, 24 hrs.)	0.21	0.32	0.04
Acid-treated catalase (pH 1.5, 24 hrs.)	0.15	0.015	0.0
Non-treated catalase	0.006	0.003	100

For comparison of the catalytic activities, E_{470}, E_{580}, and K_0 values were divided by mg. of catalase, regardless of its degradation or denaturation, contained in 0.2 ml. of the neutralized sample or diluted catalase solution applied to the experiments.

The K_0' ($mg.^{-1}$, $min.^{-1}$), value was evaluated graphically by extrapolation and expressed as percentage, taking K_0' of non-treated catalase as 100.

peroxidatic-guaiacol oxidation and oxidatic Nadi reaction are summarized along with the catalatic activity (Table I). It was demonstrated that the tryptic digest exhibited a stronger Nadi reaction than the peptic, while the peroxidatic action of the peptic digest was superior to that of the tryptic one.

Splitting of the Hematin-Protein Linkage in Acetone—After the digest had been adjusted to pH 7.4 and diluted to two-fold, the acid splitting of hematin groups from the proteinase-digested catalase in acetone was tested. The results are listed in Table II. In this experiment the insoluble materials were well mixed to make up a suspension. Hematin groups of non-treated catalase were liberated in an insignificant amount. It was evident that the acid treatment increased the amount of easily splittable hematin in acetone, which was furthermore promoted by peptic digestion. Tryptic digestion

TABLE II

Splitting of Hematin Groups in Acetone

	E_{390}/mg.	E_{405}/mg.
Peptic digest (pH 1.5, 24 hrs.)	0.060	
Tryptic digest (pH 7.4, 24 hrs.)	0.19	
Acid-treated catalase (pH 1.5, 24 hrs.)	0.54	
Non-treated catalase		0.005

Reaction mixture: 0.2 ml. of twice diluted sample at pH 7.4, 5.0 ml. acid-free redistilled acetone. The extinction coefficients at 390 mμ/cm. (E_{390}) (the digests and acid-treated catalase) and at 405 mμ/cm. (E_{405}) (catalase) were read against the blank (5.0 ml. acetone 0.2 ml. distilled water), and represented as E per mg. of catalase, regardless of its degradation or denaturation, contained in 0.2 ml. of the sample.

yielded only a small amount of easily splittable hematin.

Absorption Curve of the Digests—Both peptic and tryptic digests were adjusted to pH 7.4, diluted to twice the volume with *M*/15 phosphate buffer (pH 7.4), and the insoluble materials, if present, were removed by centrifugation. The supernatants were used for measurement of the absorption curve in the visible range. As shown in Fig. 8, the peptic digest (Fig. 8, Curve a) exhibited the bands at 390, 510 (indistinct), 540, 590 and 640 mμ, while the tryptic digest (Fig. 8, Curve b) showed the bands at 390, 510, 625 and 660 mμ.

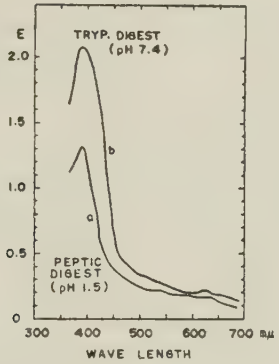


FIG. 8. Absorption curves of peptic digest (pH 1.5) and of tryptic digest (pH 7.4) for 24 hours.

The reaction mixtures were consisting of 520 mg. of catalase and 20 mg. of pepsin in 55 ml. at pH 1.5 for peptic digestion, and 520 mg. of catalase and 20 mg. of trypsin in 44.5 ml. at pH 7.4 for tryptic digestion. Digestion was continued for 24 hours at 25°. The peptic digest was adjusted to pH 7.4, and the insoluble material was centrifuged off. Tryptic digest was centrifuged too.

Paper Electrophoretic Pattern of the Digests—As shown in Fig. 9, the peptic digest, for the most part, remained at the starting line (Fig. 9, c), and corresponded to the only band of the acid-treated catalase (Fig. 9, d). The tryptic digest contained a slight amount of immobile component together with other components of higher mobilities (Fig. 9, e). As shown in Fig. 9, b, the mild peptic digestion (pH 4.0) gave a fairly amount of immobile combonent along with another component of approximately the same mobility as non-treated catalase (Fig. 9, a).

Solubility Curve of the Digests in Ammonium Sulfate Solutions—The curves of E_{275} and E_{390} *vs.* the saturation grade in percentage of the used ammonium sulfate solutions are presented in Fig. 10, a. Those curves of non-treated catalase (E_{275} and E_{405} curves) are given by the dotted lines. The E_{275} and E_{390} curves of the acid-treated catalase (A) are given in Fig. 10, b. Catalase itself was precipitated at 25–40 per cent saturation, as reported by Brown (*18*). The acid-treated catalase was far less soluble than the peptic digest

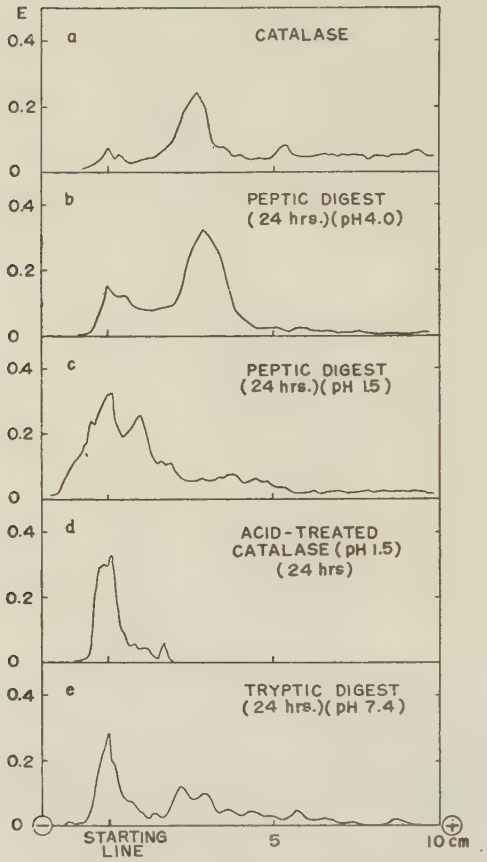
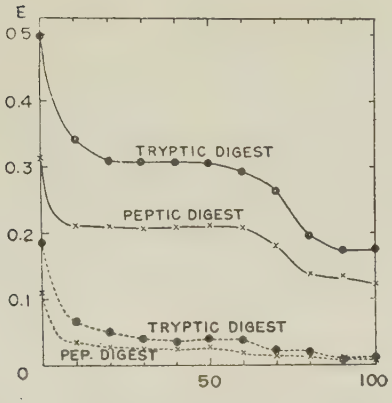


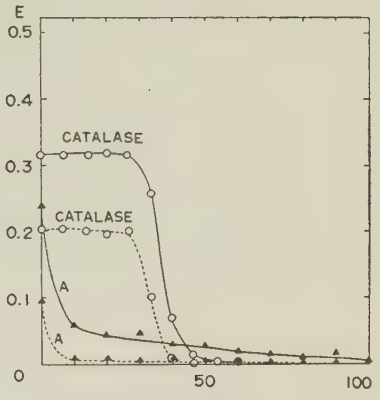
Fig. 9. Paper electrophoretic patterns of the peptic digests (pH 4.0, pH 1.5), tryptic digest (pH 7.4), acid-treated catalase (pH 1.5), and non-treated catalase.

The digestion mixtures described in Fig. 8, and the peptic digest (pH 4.0) in Fig. 5 were applied to the electrophoresis. For the acid-treated catalase see text (*Expt. III*). Conditions of paper electrophoresis: 0.1 *M* veronal buffer of pH 8.6 (μ=0.1), 150 volt, 10 mA, 5–6 hours.

(a) Non-treated catalase
(b) Peptic digest at pH 4.0, 2 hours
(c) Peptic digest at pH 1.5, 24 hours
(d) Acid-treated catalase at pH 1.5, 24 hours
(e) Tryptic digest at pH 7.4, 24 hours.

and almost completely precipitated at lower saturation (10–20 per cent). The peptic and tryptic digests, however, appeared to contain at least three components, consisting of two fractions precipitated at 20–40 and 80–90 per cent saturation, and of the one which was still soluble in a 100 per cent saturated solution.





CONCENTRATION OF $(NH_4)_2SO_4$ IN SATURATION PER CENT

FIG. 10, a. Solubility curve of the peptic digest (pH 1.5) and tryptic digest (pH 7.4).

The digests as described in Fig. 8 (see text, *Expt. III*) were used. 0.2 ml. each of the digest was mixed with 5.0 ml. of ammonium sulfate solutions from 0 to 100 per cent saturations at 10 per cent-intervals at 22–23° (room temperature) (for details see text).

× Peptic digest, ● Tryptic digest

E_{275} curve.....solid line

E_{390} curve.....dotted line

FIG. 10, b. Solubility curves of acid-treated catalase (pH 1.5) (A) and of non-treated catalase.

The solubility test was run as follows: An 0.1 ml.-portion each of stock catalase solution was mixed with 10 ml. of ammonium sulfate solution. In case of the acid-treated catalase 0.2 ml. aliquots were mixed with ammonium sulfate solutions.

▲ Acid-treated catalase

○ Non-treated catalase

E_{275} curve.....solid line

E_{390} or E_{405} curve .....dotted line

Tentative Fractionation of the Digests and Catalytic Properties of the Fractions— From the peptic digest (pH 1.5) three fractions (P_1, P_2, P_3) were separated with ammonium sulfate (40 and 85 per cent saturation). Similarly three

TABLE III

Catalytic Activities of Fractions of the Peptic and Tryptic Digests

Fraction	E_{390}	E_{275}	E_{390}/E_{275}	E_{470}/E_{275}	E_{580}/E_{275}
P_1	0.79	1.13	0.70	0.066	0.23
P_2	0.25	1.29	0.20	0.16	0.43
P_3	1.02	1.45	0.71	0.055	0. 9
T_1	0.72	1.72	0.41	0.021	0.16
T_2	0.56	1.51	0.37	0.014	0.080
T_3	0.31	0.55	0.57	0.0090	0.082

E_{470} and E_{580} represent the peroxidatic (guaiacol oxidation) and oxidatic (Nadi reaction) activities, respectively. All six fractions showed the Soret band at 390 mμ and a peak of protein in ultra-violet region at 275 mμ. For comparison of the catalytic activities, E_{470}/E_{275} and E_{580}/E_{275} are presented in the table.

fractions of the tryptic digest (pH 7.4) (T_1, T_2, T_3) were obtained by precipitation with ammonium sulfate (40 and 70 per cent saturation). Each fraction was then dialyzed and dried under reduced pressure at 0–5°.

The dried fractions were dissolved individually in 0.5 *M* NH_4OH, centrifuged, and six supernatants were tested for the Nadi reaction and peroxidatic guaiacol oxidation along with spectrophotometric measurement of E_{275} and E_{390} values. (Table III). In this table the values of E_{390}/E_{275} are given to indicate ratio of hematin groups to the protein part. All six fractions had not any measurable catalatic activity. It should be pointed out that P_2 demonstrated predominant catalytic activities among six fractions.

DISCUSSION

There have appeared a lot of reports concerning the function of the protein moiety of enzymes from a standpoint of their specificity. Sizer *et al.* (*19–21*) and Edman (*22*) attempted to modify trypsin, chymotrypsin and pepsin by tyrosinase, but they could not demonstrate the inactivation of these proteinases. The proteinase inactivation of catalase was reported by some authors (*23–25*), but they did not investigate it from a standpoint of generalization of potential catalytic abilities of hemoprotein. Recently Tsou (*9*) isolated the pepsin-modified cytochrome c, possessing the autoxidizability and ascorbic acid oxidase activity. Later, Tuppy and Palléus (*10*) purified this modified cytochrome c peptide and investigated its catalytic activity as well as its chemical structure (*11*).

The proteinase modification of catalase was reported first by the present author (*1*), who demonstrated the enhancement of peroxidatic activity for pyrogallol during the peptic inactivation of catalase.

It is quite conceivable that proteinase degradation of catalase would yield the peroxidase-like hemoprotein, since catalase bears 3 to 4 hematin groups per mole, and peroxidase only 1 hematin group per mole. These two hemoproteins are known to show differing stabilities of the protein moiety and of the hematin-protein linkage, as follows: Hematin group in catalase is split irreversibly in acidic acetone. In this connection, Lewis (*26*) studied the effect of ionic strength on the hematin liberation from catalase in acidic acetone. Horse radish peroxidase exhibits reversible recombination of its hematin group with apoprotein and, at the same time, restores its activity (*27*). As to the protein stability, catalase is so thermolabile as to be inactivated irreversibly at lower temperature (*28*), whereas peroxidase is far more thermo-stable than catalase (*29*). These differences between catalase and peroxidase suggest that the specificity of catalase may be highly associated with its hematin-protein likage as well as its protein configuration. It would, therefore, be expected that any affection upon the catalase protein might readily be reflected by the change in its catalatic activity. The experiments presented in this paper, demonstrated, in fact, the inactivation of catalase by pepsin digestion (pH 4.0) and by tryptic

digestion (pH 7.4) (*Expt. I*). It is to be noted that the peptic (pH 1.5) and tryptic (pH 7.4) digestions of catalase revealed the latent hemoprotein activities, such as peroxidatic guaiacol oxidation and oxidatic Nadi reaction. In other words, these facts are indicative that those proteinase-modified catalase preparations had gained the abilities of O_2- or H_2O_2-activation, although the activated H_2O_2 by the modified catalase molecule was not reactive at all with free H_2O_2 (Table I).

On the other hand, the modification of the catalase protein by proteinase appeared also to be reflected in the absorption curves of the Soret band (Fig. 6), which indicated at least two components: namely, the non-affected catalase (maximum at 405 mμ) and the modified-catalase (maximum at about 390 mμ). There was not confirmed any distinct isosbestic point in the absorption curves of the digest at different digestion periods, and this was probably due to the decrease in solubility in neutral solution after the digestion. The absorption peak at 390 mμ was the case with 24 hours' digests by pepsin (pH 1.5) and trypsin (pH 7.4) (see *Expt. II*, Fig. 7).

The paper electrophoretic patterns of the digests (Fig. 9) suggested that proteinase-digested catalase did not move by an electric current on filter paper, giving a band at the starting line, and remaining (non-digested) catalase was likely to situate at the position of non-treated catalase (Fig. 9, a and b). The tryptic digest contained also the immobile component along with the protein fragments of various mobilities. Since the acid-treated catalase gave its main band at the starting line, the proteinase-modified catalase could not be distinguished from the acid-treated one merely by this method. Therefore, the extraction of those bands from the filter paper was attempted for the identification of different components, but these substances were least elutable with dilute NH_4OH or sparingly eluted with phosphate buffer (pH 7–8).

Fractionation of the components in the digests was finally examined by solubility in ammonium sulfate solutions at various saturations, as shown in Fig. 10. As the digests appeared to consist mainly of three components in the solubility curves, three fractions were separated with ammonium sulfate as described in *Expt. III*. From the E_{275} and E_{390} values it was obvious that those fractions still had hematin groups attached to partially digested protein, though the hematin-protein linkage was easily split in neutral acetone (Table II).

It should be pointed out that among six fractions the second fraction of the peptic digest (P_2) was predominant in its peroxidatic and oxidatic activities (Table III). Therefore, the purification of this active fraction as the "pepsin-modified catalase" was attempted, the results of which will be reported in the following paper (*30*).

SUMMARY

1. The digestion of catalase protein by pepsin (pH 4.0) or by trypsin

(pH 7.4) lowered the catalatic activity gradually. During the digestion, peroxidatic activity for guaiacol was enhanced and increased with the digestion time.

2. During the proteinase digestion the absorption curve of the Soret band shifted to shorter wave-length by 15 mμ. The peptic digest (pH 1.5) and tryptic digest (pH 7.4) of catalase for 24 hours showed such an absorption maximum (390 mμ) of the Soret band.

3. When the digests were developed by paper electrophoresis (veronal buffer, pH 8.6, $\mu=0.1$), the peptic digest (pH 1.5) gave a main band at the starting line. Such an immobile component was alone a product of the acid-treated catalase (pH 1.5). The tryptic digest (pH 7.4) and peptic digest (pH 4.0) yielded only a small amount of the immobile band, but there remained a considerable amount of other components, including the one of the same mobility as that of catalase.

4. The solubility curves of the peptic digest (pH 1.5) and tryptic digest (pH 7.4) in ammonium sulfate, varying in concentration, indicated at least three fractions, respectively. The peptic digest was fractionated at 40 and 85 per cent saturation, and the tryptic at 40 and 70 per cent saturation. Among six fractions, P_2 (the second fraction of the peptic digest) exhibited remarkably strong activities as peroxidase (guaiacol oxidation) and indophenol oxidase (Nadi reaction).

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REFERENCES

(*1*) Anan, F.K., and Matsumura, Y., *J. Biochem.*, **43**, 103 (1956)
(*2*) Kaziro, K., *J. Jap. Biochem. Soc.*, **26**, 177 (1954); *Protein, Nucleic Acid, and Enzymes*, **1**, 1 (1956)
(*3*) Tsushima, K., *J. Biochem.*, **41**, 215 (1954); **41**, 359 (1954)
(*4*) Kikuchi, G., and Tomimura, T., *J. Biochem.*, **41**, 503 (1954)
(*5*) Suzuki, M., Tomimura, T., and Mizutani, H., *J. Biochem.*, **42**, 99 (1955)
(*6*) Kajita, A., *J. Biochem.*, **43**, 243 (1956)
(*7*) Tsushima, K., *J. Biochem.*, **43**, 13, 235 (1956)
(*8*) Tsushima, K., and Miyajima, T., *J. Biochem.*, **43**, 761 (1956)
(*9*) Tsou, C.O., *Biochem. J.*, **49**, 362, 367 (1951)
(*10*) Tuppy, H., and Palléus, S., *Acta Chem. Scand.*, **9**, 353 (1955)
(*11*) Palléus, S., Ehrenberg, A., and Tuppy, H., *Acta Chem. Scand.*, **9**, 365 (1955)
(*12*) Kajita, A., Proceeding of the 30th General Meeting of Japanese Biochemical Society, p. 47 (1957)
(*13*) Dounce, A.L., *J. Biol. Chem.*, **143**, 497 (1942)
(*14*) Kitagawa, M., and Shirakawa, M., *J. Biochem.*, **33**, 201 (1941); *J. Agr. Chem. Soc. (Japan)*, **67**, 794 (1941)
(*15*) Shirakawa, M., and Hattori, A., Standard Methods in Biochemical Researches (in Japanese), Bunkodo, Tokyo, p. 323 (1953)
(*16*) Beer, R.F., Jr., and Sizer, I.W., *J. Biol. Chem.*, **195**, 133 (1952)

(*17*) Linderstrøm, K., *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **17**, No. 4 (1927)
(*18*) Brown, G.L., *Biochem. J.*, **51**, 569 (1952)
(*19*) Sizer, I.W., *J. Biol. Chem.*, **163**, 145 (1946); **169**, 303 (1947)
(*20*) Sizer, I.W., and Fennessey, J.F., *J. Biol. Chem.*, **188**, 351 (1951)
(*21*) Haas, W.J., Sizer., I.W., and Loofbourow, J.R., *Biochim. et Biophys. Acta*, **6**, 589 (1950/51)
(*22*) Edman, P., *J. Biol. Chem.*, **168**, 367 (1947)
(*23*) Waentig, P., and Steche, O., *Z. physiol. Chem.*, **83**, 315 (1913)
(*24*) Tauber, H., and Kleiner, I.S., *Proc. Soc. Exptl. Biol. Med.*, **33**, 391 (1935)
(*25*) Sumner, J.B., and Dounce, A.L., *J. Biol. Chem.*, **127**, 439 (1939)
(*26*) Lewis, U.J., *J. Biol. Chem.*, **206**, 109 (1954)
(*27*) Gjessing, E.C., and Sumner, J.B., *Arch. Biochem.*, **1**, 1 (1941/42)
(*28*) Sizer, I.W., *J. Biol. Chem.*, **154**, 461 (1944)
(*29*) Schwimmer, W., *J. Biol. Chem.*, **154**, 487 (1944)
(*30*) Anan, F.K., *J. Biochem.*, **45**, 227 (1958)

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THE ISOLATION OF PEPSIN-MODIFIED CATALASE AND ITS CATALYTIC PROPERTIES*

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In the previous paper (*1*) it was demonstrated that the peptic and tryptic digestion of catalase resulted in progressive inactivation of catalase with time, and yielded the proteinase-modified hemoprotein compounds, catalyzing the peroxidatic guaiacol oxidation and oxidatic Nadi reaction. It is of great interest that, what configuration of the catalase protein may favor its high specificity to H_2O_2. There has not yet been attained any evidence for the free H_2O_2-combining site on the molecule of the catalase-H_2O_2 complex compound (1 mole to 1 hematin Fe atom). On the other hand, neither the hemain-linked group of catalase protein nor the amino acid sequence in vicinity of hematin group has not been clarified.

As a preliminary experiment on the chemical structure of catalase-hematin peptide, the author attempted to isolate the pepsin-digested catalase. In this paper the purification of this pepsin-modified catalase (PMC) and its some catalytic and spectroscopic properties are reported.

EXPERIMENTAL

Materials

The stock solution of crystalline beef liver catalase (1.51 g./100 ml.) in the previous paper (*1*) was used. The catalase protein was digested by crystalline pepsin (Nutritional & Biochemical Co.), which was determined to have an activity of 0.2 Anson units per mg. N.

Methods of Assay

The contents of N and Fe were estimated by the Kjeldahl and *o*-phenanthroline methods, respectively. The indophenol oxidase (the Nadi reaction),peroxidatic (guaiacol oxidation) and catalatic activities were determined by the same methods as described in the preceeding paper (*1*). The ascorbic acid oxidase activity was measured manometrically with the use of Warburg apparatus (the deatails will be described in the foot-note of Table III).

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Paper electrophoretic analysis was carried out under the same conditions as in the preceeding paper (*1*). Continuous paper electrophoresis was also applied to purify the PMC preparation, and the detail is described in the text below.

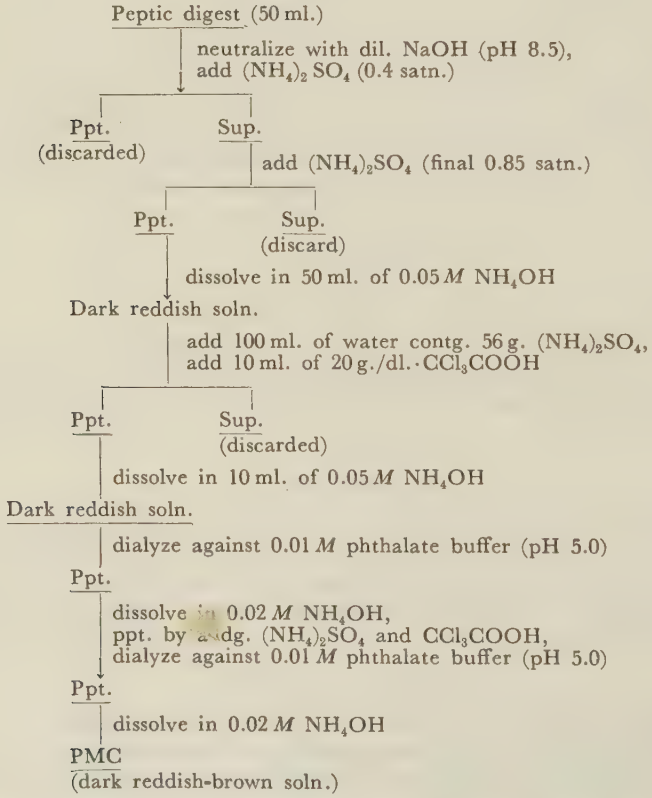
Purification of PMC

The peptic digestion of catalase was darried out as follows: Adjust 50 ml. of the stock catalase solution (750 mg.) to pH 1.5 with dilute HCl, dissolve 25 mg. of pepsin (final volume 51.7 ml.), and incubate at 25° for 24 hours. Repeat the peptic digestion (25°, 24 hours) once more by the fresh addition of 25 mg. of pepsin. The final digest looked brown with a greenish tint and slightly turbid.

The PMC was isolated, basing on the solubility curve of the peptic digest (pH 1.5, 24 hours) in the preceeding paper (*cf.* ref. (*1*), Fig. 10). In order to isolate the P_2 fraction, having high catalytic activities (*cf.* ref. (*1*), Table III), the peptic digest was first fractionated with ammonium sulfate between 0.4 and 0.85 saturations, and then purified by precipitation with trichloracetic acid and dialysis, as described by Palléus *et al.* (*2*) for the purification of pepsin-modified cytochrome c (see Scheme 1).

Scheme 1

Purification of PMC



The PMC preparation thus obtained was shown to contain 182 mg. N and 1.2 mg. Fe

per 100 ml. (average of two runs).

RESULTS

Spectroscopic Properties of PMC.—The absorption curve of PMC is given in Fig. 1., together with that of non-treated catalase (starting material). The PMC solution at pH 8 showed the bands at 275, 390 mμ and shoulder-shaped bands at 360, 500–510, 530–550, 590–610, 630–650 mμ. It was characteristic that the Soret band of PMC shifted to shorter wave-length by 15 mμ from that of catalase, and its shape was not so sharp as that of catalase, but three was observed a big shoulder band at about 360 mμ in PMC.

The absorption curves of PMC in the visible range on the addition of KCN and $Na_2S_2O_4$ are shown in Fig. 2. On adding 2–3 drops of 1 per

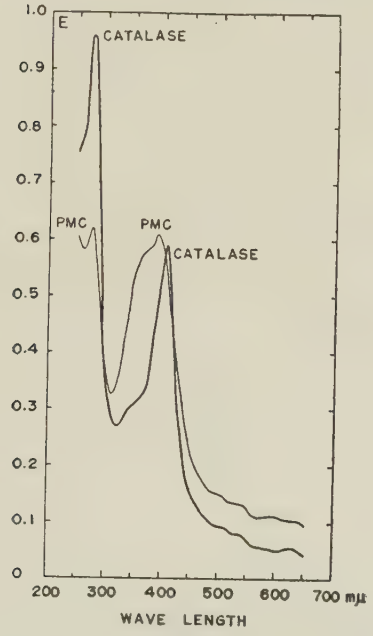


Fig. 1. Absorption curves of PMC and catalase.

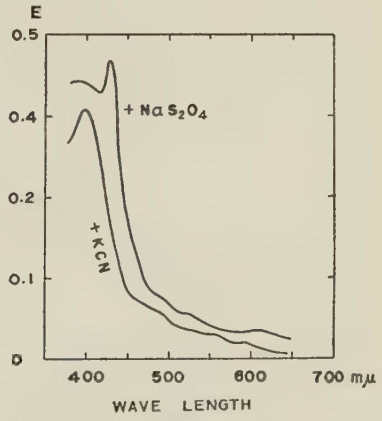


Fig. 2. Absorption curves of PMC on the addition of KCN and $Na_2S_2O_4$.

cent $Na_2S_2O_4$ to the PMC solution the Soret band was converted to dual peaks: a sharp peak at 425mμ and a broad band at 380–400 mμ, but it could not be conclusively demonstrated that the reduction of PMC hematin Fe readily occurred, and this characteristic soret band did not return to the original PMC band even by aearation for 30 minutes. The addition of 2–3 drops of 1 per cent KCN to the PMC solution shifted the Soret band at 480–500, 560, 590 mμ. The latter two bands were also observed with KCN-catalase.

Paper Electrophoretic Pattern of PMC—The densitometric patterns of paper electrophoresis of PMC and non-treated catalase are shown in Fig. 3, a and b, respectively. A pattern of the peptic digest prior to the purification of PMC is also given in Fig. 3, c. It appeared that PMC was a non-moving component contained in the peptic digest. As the acid-denatured catalase gave a sole band at the starting line (*cf.* preceeding paper (*1*), Fig. 8, c), PMC was not distinguishable from the acid-denatured catalase by paper

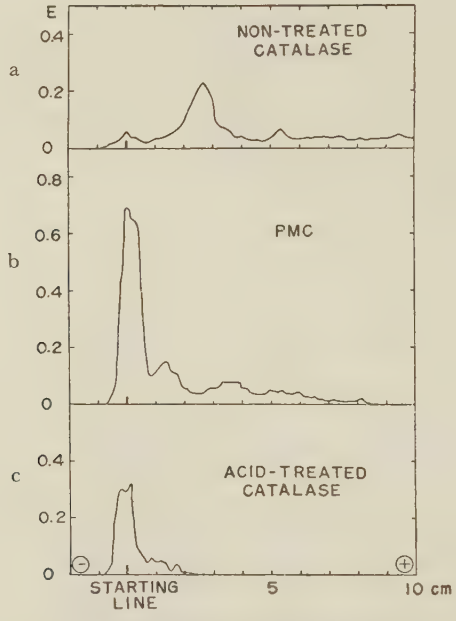


Fig. 3. Paper electrophoretic patterns of PMC, catalase and acid-treated catalase.

Fig. 3, a. .....Non-treated catalase.
Fig. 3, b. .....PMC
Fig. 3, c. .....Acid-treated catalase
(pH 1.5, 24 hrs.)

Conditions: 0.1 *M* veronal buffer (pH 8.6), μ 0.1, 150 v., 10 mA., Bromphenol blue staining.

electrophoretic analysis alone. The extraction of those bands from the filter paper with 0.05–0.1 *M* NH_4OH or *M*/15 phosphate buffer (pH 7.4) was not successful, as it was the case with the peptic and tryptic digests (*1*).

Catalytic Properties of PMC—

(a) *The Peroxidatic Oxidation of Guaiacol*: PMC exhibited to catalyze the peroxidatic oxidation of guaiacol by H_2O_2, which was strongly inhibited by 10^{-3}*M* KCN, and considerably by 10^{-3} *M* NaN_3 or NaF. (see Table I). The optimum pH of the peroxidatic activity of PMC was found at pH 5–6 (Fig. 4).

TABLE I

The Peroxidatic Activity of PMC

Addition	Concn. (*M*)	E_{470}/mg.*	E_{470}/E_{275}
None		0.72	0.45
KCN	10^{-3}	0.23	0.14
NaN_3	10^{-3}	0.32	0.19
NaF	10^{-3}	0.33	0.19

Reaction mixture: 0.2 *M* acetate buffer (pH 5.0), 2.3 ml.; 1 per cent guaiacol, 1.0ml.; 0.5 per cent H_2O_2, 1.0ml.; distilled water or $10^{-2}M$ inhibitor, 0.5 ml.; PMC solution, 0.2 ml. After standing for 30 minutes at room temperature, dilute to 10.0 ml., and read E_{470} ($cm.^{-1}$). The PMC solution contained 182 mg. N per 100 ml., and showed the value of $E_{275}/cm.=0.62$. * PMC in mg. in 0.2 ml.

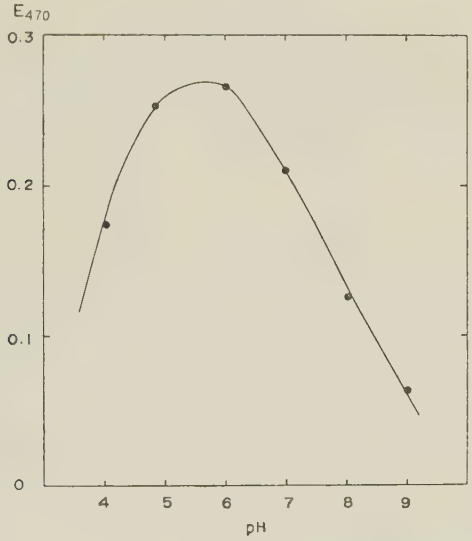


FIG. 4. The peroxidatic activity of PMC and pH.
Reaction mixture: 0.1*M* buffer (acetate buffer, pH 4–5; phosphate buffer pH 7–9) 4.0 ml., 1 per cent guaiacol, 1.0 ml., 0.5 per cent H_2O_2, and 1.0 ml., PMC solution, 0.2 ml. After standing for 30 minutes at room temperature, dilute to 10.0 ml. and read E_{470} ($cm.^{-1}$).

(b) *The Indophenol Oxidase (Nadi Reaction) Activity*: PMC was capable of catalyzing the Nadi reaction too, as shown in Table II. KCN, NaN_3 and NaF were also inhibitory.

(c) *Ascorbic Acid Oxidase Activity*: PMC was catalytically active for the aerobic oxidation of L-ascorbic acid at pH 6.8, and this activity was evidently inhibited by $10^{-3}M$ KCN as shown in Table III. PMC showed, however, much lower Q_{O_2} value for L-ascorbic acid than the pepsin-modified

Table II

The Indophenol Oxidase Activity of PMC

Addition	Concn. (*M*)	E_{580}/mg.*	E_{580}/E_{275}
None		0.61	0.35
KCN	10^{-3}	0.17	0.22
NaN_3	10^{-3}	0.22	0.13
NaF	10^{-3}	0.29	0.17

Reaction mixture: *M*/15 phosphate buffer (pH 7.2), 1.7ml., 0.05 *M* alcoholic α-naphthol, 0.5 ml.; 0.05*M* dimethyl-*p*-phenylenediamine hydrochloride, 0.5ml.; PMC, 0.2 ml.; distilled water or inhibitor ($10^{-2}M$), 0.3 ml. After standing for 5 minutes, add 3.2 ml. of 95 per cent ethanol, and read E_{580} ($cm.^{-1}$) against the blank in which PMC was replaced by 0.2 ml. of water. * PMC in mg. in 0.2 ml.

Table III

The Ascorbic Acid Oxidase Activity of PMC

PMC contd. (*ml.*)	In absence of KCN	In presence of KCN ($10^{-3}M$)	Inhibition
	Q_{O_2}	Q_{O_2}	%
0.10	302	135	55.3
0.05	266	98	63.1
0.025	324	126	61.1
Average	297	120	59.6

Reaction mixture: *M*/15 phosphate buffer (pH 6.8), 1.0 ml., PMC diluted solution at 1 : 10, 1 : 20, 1 : 40, with the buffer, 1.0 ml., in main vessel (for control use the buffer, 2.0ml.); 0.01*M* L-ascorbic acid, 0.5ml. in side arm. O_2-uptake was read for 30 minutes, and the first 10 minutes' O_2-uptake after reducing that of the control was used for calculation of Q_{O_2} values. In inhibition expts., 1 ml. of 0.0025 *M* KCN solution in the buffer was used (final concentration, $10^{-3}M$) in stead of the buffer in the control.

cytochrome c preparation of Tsou (*3*)

Continuous Paper Electrophoretic Pattern of PMC—Because of scarce solubility of PMC in either upper or lower layer of the butanol-acetic acid-water mixture (4: 1: 5), the column chromatographic separation of PMC by such a solvent that was used for purification of the pepsin-modified cytochrome c (*2, 3*) was not applicable. In order to examine the homogeneity of PMC and if possible, to purify PMC furthermore, the PMC preparation was tested by continuous paper electrophoresis, as follows: A filter paper (Toyo Roshi, No. 51, 30×20cm.) was placed on an inclining plate at about 60°, and kept standing to soak up 0.01 *M* phosphate buffer (pH 7.0). PMC solution was diffused to the filter paper from a small glass cup through a strip cut out of the filter paper as shown in Fig. 5, a, and electrophoresis was run at 150 volt, 10 mA., for about 36 hours. The filter paper was dried and stained

with bromphenol blue (see Fig. 5, a). It was noticed that before staining the brown colored substances gave their main traces, flowing down almost straightly. At the same time, the effluents were collected in test tubes,

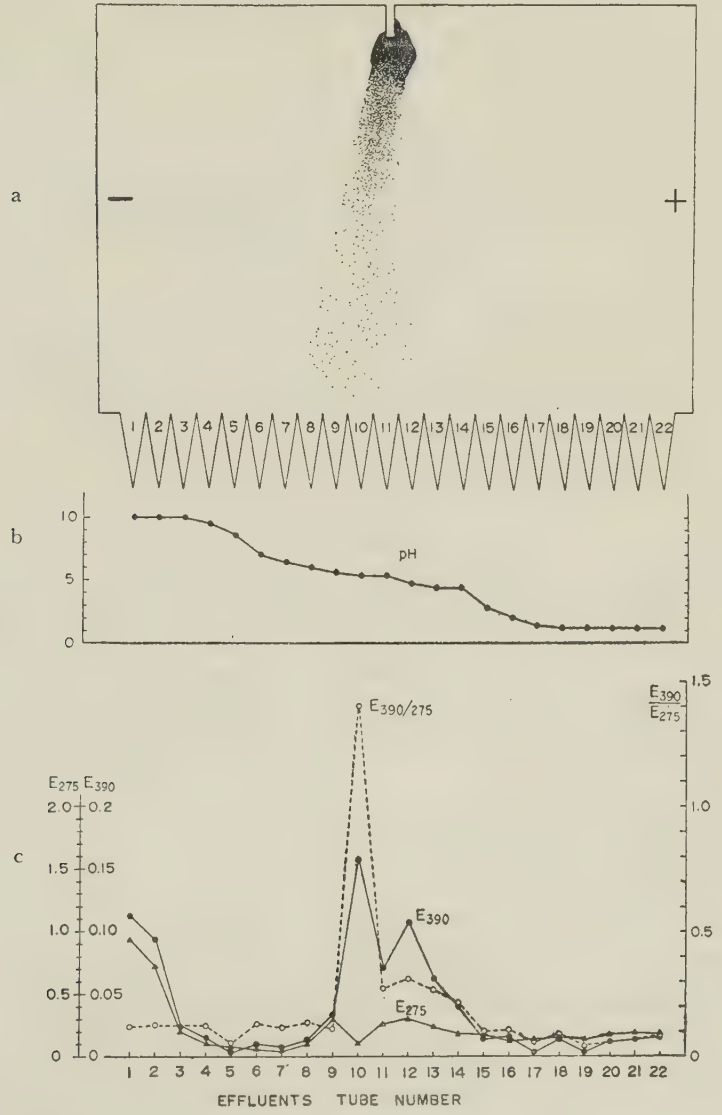


Fig. 5. a, b, c.

checked for pH, (Fig. 5, b) then adjusted to pH 7.0 with dilute HCl or NaOH, diluted exactly to 10.0 ml. with *M*/15 phosphate buffer (pH 7.0), and examined for the E_{275} and E_{390} values as well as the peroxidatic activity

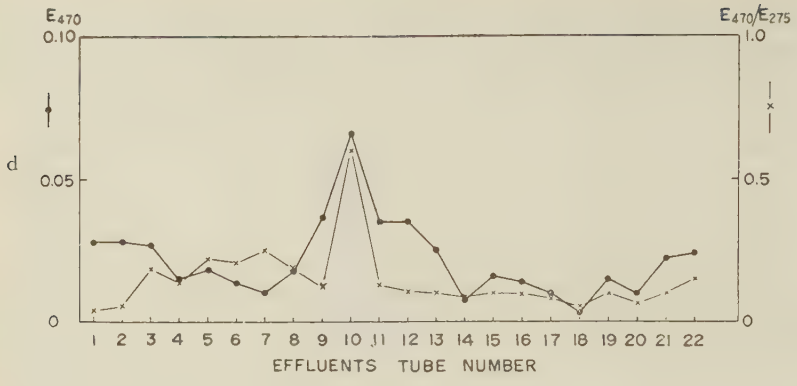


Fig. 5. d.

(guaiacol oxidation). The results are shown in Fig. 5, c, in which the E_{390}/E_{275} curve is also given. It should be pointed out that the effluent of the tube No. 10 showed a markedly high content of hematin group (E_{390}/E_{275}), and at the same time, a predominantly strong peroxidatic activity (E_{470}/E_{275}) as shown in Fig. 5, d.

DISCUSSION

In this paper a catalytically active PMC fraction was isolated, basing on the solubility curve of the peptic digest of catalase (*cf.* preceeding paper (*1*)). The PMC preparation showed a main band in the paper electrophoretic pattern (Fig. 3), but the continuous paper electrophoretic analysis of PMC indicated that the PMC fraction purified by the procedures as shown in Scheme 1 was not strictly homogeneous, but it was consisting of approximately homogeneous, partially digested hemoproteins of catalase as suggested in Fig. 5.

As to the chemical properties of PMC it should be pointed out that the PMC preparation appeared to bear still the protein part of a fairly high molecular weight, attaching the hematin group (s), as obviously shown by the E_{275}/E_{390} value. By a simple calculation from E_{275}/E_{390} of PMC (1.02) and E_{275}/E_{405} (1.53), PMC was still holding 64 per cent of the protein part of original catalase per hematin group. On the other hand, the chemical analysis for N and Fe contents of catalase solution (N/Fe=195) and of the PMC solution (N/Fe=151) led ta a 32 per cent loss of the protein part in disintergration of catalase to PMC. In fact, PMC was shown to be insoluble in a butanol-acetic acid-water mixture (4: 1: 5) on attempting the column chromatography with Hyflo Supercel. Since amino acid composition and molecular weight of PMC were left in future experiments, it was not confirmed, whether or not the PMC molecule (s) would still bear 3 or 4 hematin groups per mole. The PMC preparation presented in this paper was not so-called "hemopeptide" or "iron porphyrin peptide" like the pepsin-

modified cytochrome c preparations of Tsou (*3*), Tuppy *et al.* (*4*), and Minakami *et al.* (*5*). For analysis of the chemical structure of catalase surrounding its hematin group (s), the PMC preparation should be more deeply degraded.

The peptic attack of the catalase protein was suggestively reflected in the absorption spectra of PMC (Fig. 1), though neither hydrolyzed bond nor liberated protein fragment was analyzed here. PMC gave a peculiar Soret band together with a new band at about 360 mμ. The reactivity of PMC with KCN and $Na_2S_2O_4$ was also confirmed not only by the change in the absorption curves on adding them (Fig. 2) but also by the KCN inhibition of the peroxidatic and oxidatic activities of PMC (Tables I and II).

The isolation of more purified and more catalytically active PMC fraction was shown to be possible from the continuous paper electrophoretic pattern (Fig. 5). It was noticed that, of 22 effluents, the No. 10 fraction exhibited stronger peroxidatic activity ($E_{470}/E_{275}=1.2$) than non-fractionated PMC ($E_{470}/E_{275}=0.34$), and higher hematin content ($E_{390}/E_{275}=1.40$) than PMC ($E_{390}/E_{275}=0.98$).

SUMMARY

1. From the peptic digest of catalase at pH 1.5 the pepsin-modified catalase (PMC), possessing generalized potential catalytic abilities as hemoprotein, was isolated. The purification of PMC consisted of ammonium sulfate fractionation between 0.40 and 0.85 saturation, precipitation with ammonium sulfate plus trichloracetic acid, and dialysis at pH 5.0, successively

2. The PMC preparation showed a sole band at the starting line it its paper electrophoretic pattern (veronal buffer, pH 8.6, $\mu=0.1$). PMC demonstrated a peculiar Soret band (the max. at 390 mμ), which was evidently changed on adding KCN or $Na_2S_2O_4$.

3. PMC exhibited fairly strong activities of the peroxidatic guaiacol oxidation, and the oxidatic Nadi reaction (indophenol oxidase activity). PMC could catalyze aerobic oxidation of L-ascorbic acid (Q_{O_2}=average 297). These catalytic activities of PMC were strongly inhibited by $10^{-3}M$ KCN, considerably by $10^{-3}M$ NaN_3 and NaF.

4. PMC still had the protein part of catalase of a high molecular weight, and the loss of protein part in disintegration of catalase to PMC was calculated as about 36 per cent per hematin group from the values of E_{275}/E_{390} and $E_{275}/_{405}$, and 32 per cent from N and Fe contents.

5. Continuous paper electrophoresis of PMC yielded more active PMC fraction, which demonstrated markedly stronger peroxidatic activity and higher hematin content.

The author wishes to express his thanks for helpful advises of Prof. S. Miyamoto and Prof. K. Kaziro and for technical assistance of Miss H. Suzuki.

REFERENCES

(*1*) Anan, F.K., *J. Biochem.*, **45**, 211 (1958)
(*2*) Tsuo, C.L., *Biochem., J.*, **49**, 362, 367 (1951)
(*3*) Palléus, S., Ehrenberg, A., and Tuppy, H., *Acta Chem. Scand.*, **9**, 365 (1955)
(*4*) Tuppy, H., and Palléus, S., *Acta Chem. Scand.*, **9**, 353 (1955)
(*5*) Minakami, S., Titani, K., and Ishikura, H., *J. Biochem.*, **44**, 535 (1957)

The Journal of Biochemistry, Vol. 45, No. 4, 1958

INCORPORATION IN VITRO OF LABELED SULFURIC ACID INTO RAT LIVER HEPARIN*

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(Received for publication, September 2, 1957)

During the course of studies on conjugation *in vitro* of phenols with S^{35}-labeled inorganic sulfate by liver slices (*1*), it was found that the liver proteins coagulated by trichloracetic acid contained slight but distinct radioactivity even when the incubation was carried out without phenols. Further studies suggested that inorganic sulfate was incorporated into heparin, as reported in a previous communication (*2*). The outline of the experiment was as follows: the liver slices and homogenate were incubated with S^{35}-labeled inorganic sulfate under oxygen and deproteinized with trichloracetic acid; the precipitate was extracted with chloroform-methanol, a small amount of heparin was added as a carrier and the heparin fraction was obtained by precipitation with cadmium chloride as described by Scott and Charles (*3*); this contained a distinct radioactivity, while no activity was detected in the fraction when the incubation was carried out under nitrogen.

More recently, Bassiouni (*4*) has devised a paper electrophoretic isolation of heparin and Kerby (*5*) a paperchromatographic separation of heparin and chondroitin sulfuric acid. Since chromatographic methods are more suitable to avoid the possible contamination with radioactive impurities, it seemed worth while to carry out the experiments adopting the techniques developed by Bassiouni and Kerby.

EXPERIMENTAL

Experiments with Slices—The rat liver was cut into slices in an ice-cold sulfate-free Ringer-phosphate solution, and 0.3 g. of the slices was transferred to each reaction vessel containing 0.5 ml. of succinate-Ringer-phosphate solution and 0.05 ml. of S^{35}-labeled sulfate solution (about 20 μc. carrier-free). The vessels were incubated in a thermostat at 37.5° under oxygen for 2 hours with constant shaking (*1*). At the end of the incubation 100 μg. of heparin were added to each vessel as a carriel and reisolated according to Bassiouni (*4*) with a slight modification, toluidine blue being used as a precipitant of heparin.

* The subject matter of this paper has appeared, written in Japanese, in the Jounal of Japanese Biochemical Society, Vol. 27, p. 672-674 (1955).

The slices were homogenized together with the reaction mixture and additional 7 ml. of water in the apparatus of Potter and Elvehjem (*6*). The homogenate was made alkaline by the addition of 0.5 ml. of 2 *N* NaOH, and heated at 50° for 30 minutes; 6.5 g. of ammonium sulfate were added and the homogenate was shaken to complete solution. After 5 minutes the mixture was heated again until the temperature reached 75° in a boiling water bath and filtered quickly. The filtrate was received in a mixture of 1 ml. of water and 1 ml. of a 0.1 per cent solution of toluidine blue and allowed to stand for an hour with occasional shaking. The precipitate which formed was collected by centrifugation, washed with 5 ml. of 0.01 *N* HCl and with 2 ml. of water, resuspended in 0.25 ml. of formamide, and the suspension was heated at 50° for several minutes and centrifuged again.

The supernatant liquid was transferred to another tube and 0.25 ml. of saturated sodium chloride solution and 2ml. of methanol were added. The precipitated heparin was centrifuged, redissolved in 0.25 ml. of saturated sodium chloride solution, and reprecipitated by addition of 0.25 ml. of water and 2 ml. of methanol. This will be called "formamide soluble fraction".

Experiments with Liver Extract—The liver was homogenized with an equal volume of a mixture (8 : 2 in volume) of 0.154 *M* potassium chloride and magnesium chloride solutions, and the supernatant fluid was obtained by centrifuging at about $10{,}000 \times g$. Each reaction vessel received 0.3 ml. of the extract, 3 mg. of potassium adenosine triphosphate (ATP) and about 20 μc. of radioactive inorganic sulfate. The incubation was carried out at 37.5° under air for 2 hours. Further steps were the same as in the experiment with liver slices, except that the homogenization after the incubation was omitted.

Paper Chromatography—The reprecipitated "formamide soluble fraction" was dissolved in 0.5 ml. of water and its small portion was placed on strips of Whatman No. 1 filter paper. A one-dimensional ascending chromatogram was developed with supernatant fluid from a mixture of 10 ml. of *n*-propanol and 10 ml. of *M*/40 phosphate buffer pH 6.4 at room temperature for 16 hours. This was a modification of the method of Kerby (*5*). In the original method, the chromatogram is run is 25 percent propanol in *M*/15 phosphate bufier pH 6.4 for 33 hours at 4°.

Heparin could be easily located by dipping in 0.5 per cent solution of toluidine blue, resulting in a spindle-shaped violet spot with an R_f value of about 0.5 on a blue background. Chondroitin sulfate gave a similar spot with lower R_f value.

The sediment remaining undissolved in formamide; which will be called "formamide insoluble fraction", was taken up in a small amount of water, to its half portion were added 30 μg. of heparin, and the mixture was chromatographed in the same way as described avove.

Radioautography—The colored chromatogram was placed in contact with Fuji No-Screen X-ray film No. 400 for about one week to obtain the radioautogram (*7*). If the isolation procedure was carried out prior to incubation practically no radioactivity was demonstrated on the chromatogram, probably because of an extensive dilution of the isotope by the addition of a large amount of ammonium sulfate in the course of preparation. Consequently, if any radioactive spot is obtained on the chromatogram it may be concluded that a biological sulfatation has taken place undoubtedly during the incubation with liver preparation.

RESULTS

As shown in Fig. 1, when the liver slices were incubated with radioactive sulfate and the "formamide soluble fraction" was submitted to paper-

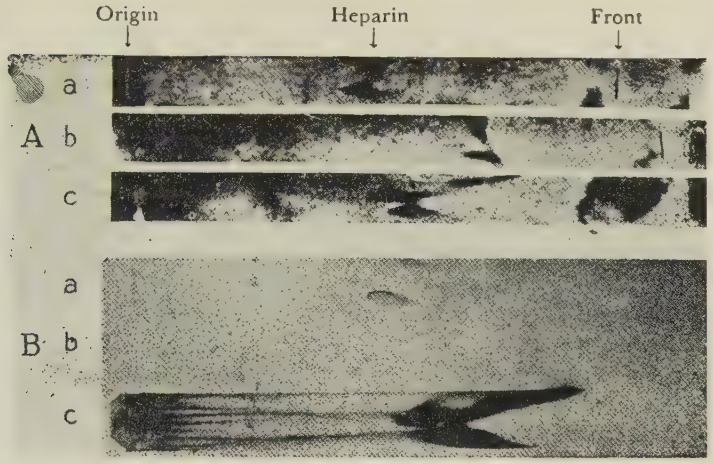

FIG. 1. Incorporation of S^{35}-sulfate into "formamide soluble fraction".
A, paper chromatograms sprayed with toluidine blue.
B, radioautograms obtained from A, a little enlarged.

Reaction mixtures incubated at 37.5° for 2 hours under air in a and b, and under oxygen in c. a, containing 0.3 ml. of supernatant fluid of liver homogenate, 3 mg. of ATP and S^{35}-sulfuric acid (carrier-free). After incubation, following the addition of 100 μg. of heparin, "formamide soluble fraction" was prepared as described in the text. b, the same as in a except that 50 μg. of heparin were added before the incubation. c, containing 0.3 g. of liver slices, 0.5 ml. of succinate-Ringer-phosphate solution and S^{35}-sulfuric acid. After incubation, following the addition of 100 μg. of heparin, "formamide soluble fraction" was prepared as described in the text.

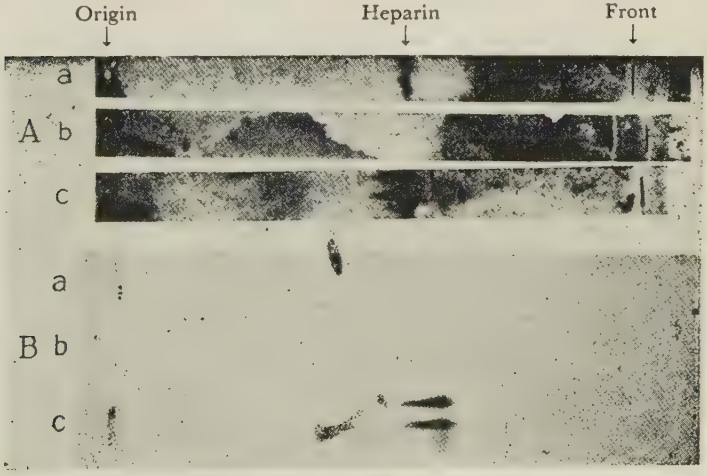

FIG. 2. Incorporation of S^{35}-sulfuric acid into "formamide insoluble fraction". Conditions were the same as in Fig. 1.

chromatography, a violet spot corresponding to heparin appeared by dipping in toluidine blue and the radioautographic shading coincided in shape and height with this heparin spot. The experiments with liver extract gave a similar spot of heparin which possessed a much weaker radioactivity than in the case of liver slices. When 50 μg. of heparin were added to the liver extract prior to the incubation, no shading was obsereved on the radioautogram. This seems to be due to that inorganic sulfate which is liberated from the added heparin dilutes the isotopic inorganic sulfate in the medium.

There appeared another spot of blue violet color near the orgin, which took up the radioactive isotope. The incorporation of S^{35}-sulfate in this substance was more active for the liver slices than for the liver extract as in the case of heparin.

The paper chromatograms for the "formamide insoluble fraction" showed a similar spot corresponding to heparin and a blue-violet one near the origin, both of which gave shadings on the radioautograms as shown in Fig. 2.

DISCUSSION

The experimental results led the authors to a conclusion that inorganic sulfuric acid is incorporated into rat liver heparin *in vitro*. The incorporation of sulfate into chondroitin sulfuric acid of cartilage has been reported by Boström (*8*). More recently, Suzuki (*9*) has demonstrated that the mucous cells of *Charonia lampas* (L.) (Boshu-hora) took up sulfate ion labeled with radioactive sulfur in sea water into charonin sulfuric acid. The biological sulfatation of mucopolysaccharide with inorganic sulfate seems to be a generally occurring process whatever the reaction mechanism may be.

It is noteworthy that a slight but distinct incorporation of S^{35}-sulfate into heparin has been observed by an incubation with the cell-free supernatant fluid of liver homogenate in the presence of ATP. This fact suggests that the enzyme system for the sulfatation of heparin is in the supernatant fraction and the energy for the reaction is supplied from ATP. Further studies are needed to establish the best condition for the reaction with the soluble enzyme preparations.

Of some interest is the substance with a small R_f value which incorporates S^{35}-sulfate ion in some extent. This substance is precipitated together with heparin by treating the solution with toluidine blue but distinctly different in its properties from heparin and chondroitin sulfuric acid. This component may account for the different activities of heparin preparations from different sources as described by Jacques, Waters and Charles (*10*).

SUMMARY

Rat liver slices incorporated S^{35}-labeled sulfate into haperin *in vitro*. The incorporation of sulfate was also demonstrated with the supernatant

fluid of rat liver homogenate in the presence of ATP though in less extent.

Another unknown substance was found which incorporated inorganic sulfate.

REFERENCES

(*1*) Sato, T., Suzuki, T., and Yoshikawa, H., *J. Biochem.*, **40**, 663 (1953)
(*2*) Sato, T., Suzuki, T., and Yoshikawa, H., Proceeding of the 25th Annual Meeting of the Japanese Biochemical Society, in *J. Japanese Biochem. Soc.*, **25**, 63 (1953)
(*3*) Scott, D.A., and Charles, A.F., *J. Biol. Chem.*, **102**, 437 (1933)
(*4*) Bassiouni, M., *J. Clin. Path.*, **6**, 39 (1953); **7**, 330 (1953)
(*5*) Kerby, G.P., *Proc. Soc. Exptl. Biol. Med.*, **82**, 263 (1953)
(*6*) Potter, V.R., and Elvehjem, C.A., *J. Biol. Chem.*, **114**, 495 (1936)
(*7*) Sato, T., Yamada, M., Suzuki, T., Fukuyama, T., and Yoshikawa, H., *J. Biochem.*, **43**, 25 (1956)
(*8*) Boström, H., and Mansson, B., *J. Biol. Chem.*, **19**, 483 (1952)
(*9*) Suzuki, S., *J. Biochem.*, **42**, 691 (1956)
(*10*) Jacqus, L.B., Waters, E.T., and Charles, A.F., *J. Biol. Chem.*, **144**, 229 (1942)

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STUDIES ON THE DENATURATION OF ENZYMES

II. EFFECTS OF INORGANIC AND ORGANIC SUBSTANCES ON HEAT-INACTIVATION OF SWEET POTATO β-AMYLASE

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(Received for publicaton, November 1, 1957)

Many examples are known that the heat-stability of enzymes, or of proteins is affected by their own impurities or other substances, including coenzymes, substrates, neutral salts, inorganic and organic ions and other substances (*1*).

It has been well known that α- or dextrinizing amylases are stabilized by calcium or other alkaline earthes, alkalis, halogenides and other ions (*e. g., 2–5*). On the other hand, it has been reported that β- or saccharifying amylases, are instabilized in the presence of certain salts (*e.g., 6, 7*). Concerning the mechanisms of these effects of ions, however, only a few studies on α-amylases have so far been reported (*5, 8*).

In the present study, effects of several inorganic and organic substances upon heat-inactivation of sweet potato β-amylase will be treated.

EXPERIMENTAL

The method of heating and measurement of enzyme activity of the sweet potato β-amylase preparation were the same as in the previous report (*9*), with the exception noted below. Sweet potato was sliced, air dried, powdered (80 to 100 mesh) and extracted with water, from which was prepared purified paste in usual manner. Sweet potato lost practically no amylase activity by air drying, by which the efficiency of extraction rather increased. Further, dried sweet potato has the advantage that it can be stored for a long period to be used as occasion demands*.

Temperature of inactivation was 63±0.1° at pH 5.4, which is the most stable pH of

* Giri *et al.* (*10, 11*) had used dried meal of sweet potato for extraction of amylase. They had extracted the dried meal with water in the presence of toluene for 12 hours or longer, and from the extract amylase was precipitated with alcohol, and was purified by dialysis. In our experiments, however, 1 kg. of dried powder, for example, was stirred in 2.5–3 liters of water at room temperature, and then immediately filtered on Buchner's funnel. The residue was extracted again with 1–1.2 liver of water. Thus, about 2.5–3.5 liters of extract was obtained, which contained about 80 per cent of extractable amylase. Sweet potato β-amylase preparation precipitated with alcohol was unstable in a dried condition.

Dried material appears to have some advantages for purification of the amylase, investigations of which are in progress in our laboratory.

this enzyme (*9*)*. The residual activities were measured at 40° by means of micro-Bertrand procedure, and were represented in percentage against the value of control experiment at zero time heating.

RESULTS

I. Influences of Inorganic Ions upon Heat-Inactivation of Sweet Potato β-Amylase—Several inorganic salts were added to amylase in acetate buffer, and their influences upon heat-inactivation rate of the enzyme were investigated.

At an order of $10^{-3}\,M$ concentration, inorganic anions and cations tested here had almost no effect upon heat-inactivation of β-amylase, or increased slightly the inactivation rate, if any. At $10^{-2}\,M$, however, all of these increased the inactivation rate more or less. The results are shown in Fig. 1, although data at $10^{-3}\,M$ of ions were omitted. In the case of halogenides, fluoride was exceptionally sodium salt, since pure pottasium salt was not available. It had been confirmed, however, that there was practically no

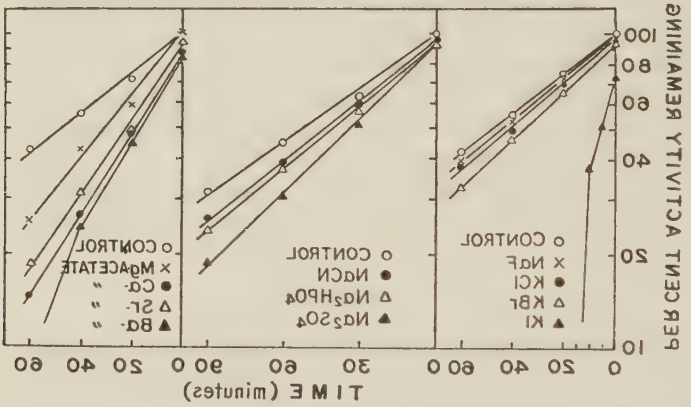

Fig. 1. Influences of inorganic ions upon heat-inactivation of sweet potato β-amylase.

Enzyme solution containing $10^{-2}\,M$ ion and $M/20$ acetate buffer (pH 5.4) was heated at 63±0.1°. Activity measurement: heated enzyme solution 0.5 ml., $M/2$ acetate buffer (pH 5.4) 0.1 ml., and 5 per cent soluble starch 1.0 ml., in total volume 2.0 ml.; 40°. As to cations of halogenides used, see text.

difference in the effect between sodium and pottasium in this experimental condition, using their chlorides. Although it was impossible to compare directly the extents of the effects of ions having different control curve from each other, it is noticeable that the greater the atomic number of halogen,

* In 1934 Giri had reported already the studies on the heat inactivation of sweet potato amylase precipitated with alcohol and dialyzed, in the papar cited above (*10*). He had reported that the process of heat inactivation of the amylase followed a unimolecular course, and the enzyme was most stable in the region of pH 5.4 and 6.0.

the greater the effect to increase the heat-inactivation rate of amylase. Similar tendency was observed with alkaline earth ions.

II. Influences of Concentrations of Inorganic Ions—The influences of the concentrations of sodium-chloride and sulfate, and calcium acetate upon the heat-inactivation of amylase were investigated. The results are shown in

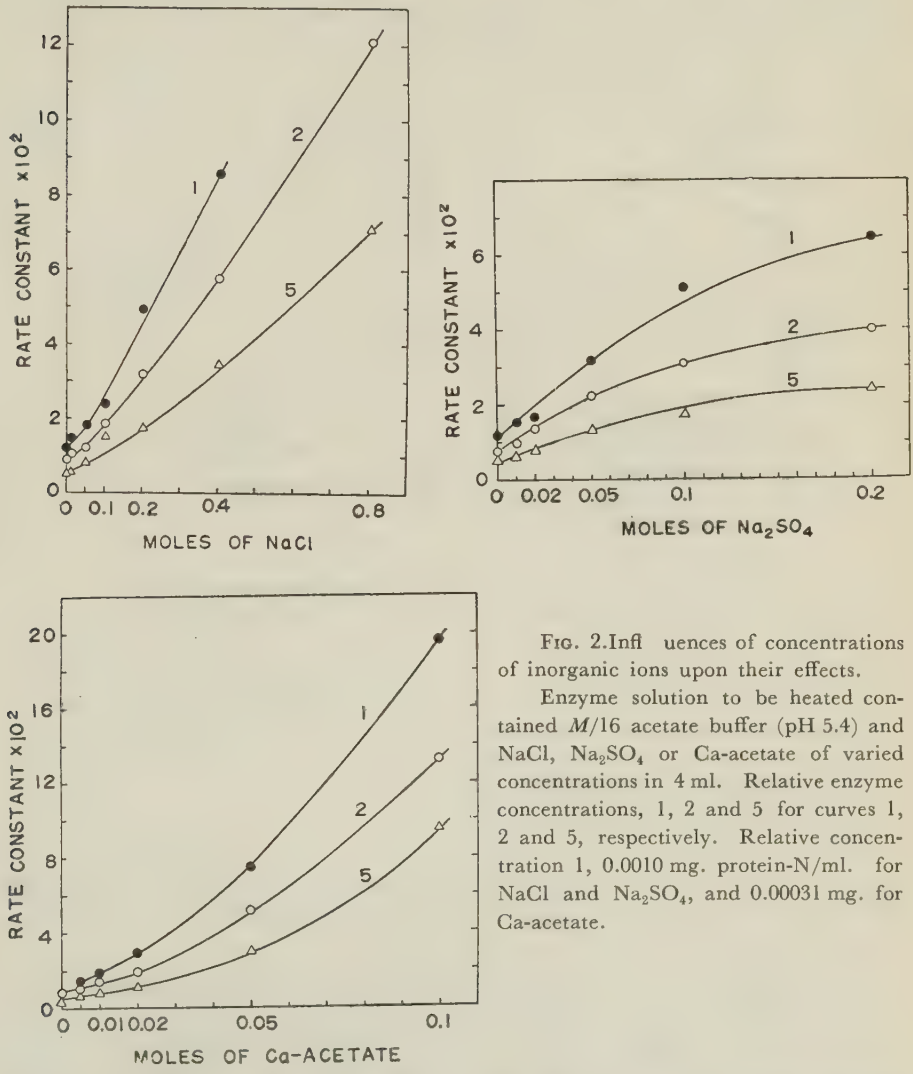

Fig. 2.Infl uences of concentrations of inorganic ions upon their effects.

Enzyme solution to be heated contained *M*/16 acetate buffer (pH 5.4) and NaCl, Na_2SO_4 or Ca-acetate of varied concentrations in 4 ml. Relative enzyme concentrations, 1, 2 and 5 for curves 1, 2 and 5, respectively. Relative concentration 1, 0.0010 mg. protein-N/ml. for NaCl and Na_2SO_4, and 0.00031 mg. for Ca-acetate.

Fig. 2. In these experiments, enzyme was used at three different concentrations. In all these experiments, with the increase of the concentration of ions, the instabilizing effect increased. It is remarkale, however, that the curve of sulfate is different from that of chloride and calcium salt.

III. Influences of Organic Acids—Sweet potato β-amylase in buffer of formate, acetate, succinate malate, tartrate or citrate was heated, and the inactivation rates of enzyme were compared at pH 5.4. No appreciable change of pH was recognized during heating.

Acids with equal number of carboxylic groups have approximatery the same effect, and the greater the number of carboxylic groups, the greater the rate of heat-inactivation of amylase, as illustrated in Fig. 3. Then the

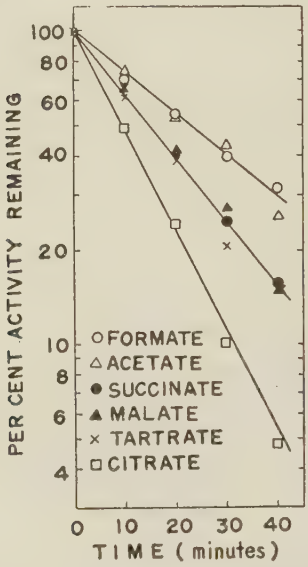

Fig. 3. Heat-inactivation rates in the presence of organic acids.

Formic acid, as sodium salt, was adjusted to pH 5.4 with a few drop of dilute acetic acid; other acids, as free acid solution, were neutralized to pH 5.4 with *N* NaOH; final concentration *M*/8.

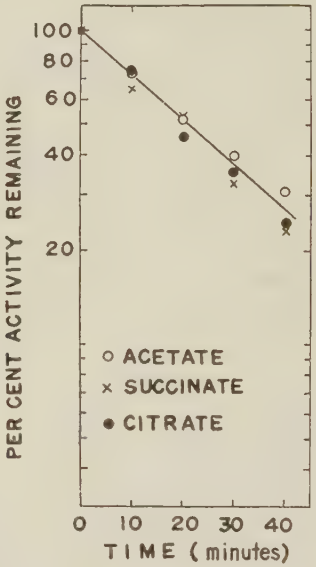

Fig. 4. Heat-inactivation rates in equi-normal solution of organic acids.

Acid concentrations, as follows: acetic *M*/8, succinic *M*/16, citric *M*/24.

effects of acetate, succinate and citrate with relative mole concentrations of 3, 1.5 and 1, respectively, were compared. Their effects were almost the same as illustrated in Fig. 4.

From these results, it appeard that the effects of these organic acids upon heat-inactivation of the β-amylase depend upon the concentration of carboxylic groups, and the higher the concentration, the faster the inactivation. And the rate appeared to be independent of the structures of the organic anions.

The ionic strengths of acetate, succinate and citrate in the latter exderiment were 0.10, 0.14 and 0.21, respectively. But no experiment at equal ionic strength was carried out.

IV. Influences of Amino Acids—Monoamino-monocarboxylic acids such as glycine, alanine, valine and serine, have little or no effect upon heat-inactivation of sweet potato β-amylase at $5\times10^{-2}M$ (experimental data are omitted). Many other amino acids could not be tested, because of their slighter solubility at pH 5.4.

Glycine, which have comparatively higher solubility in this experimental condition, however, had some protective effect at as high as $1\,M$ as illustrated in Fig. 5. While, alanine had no effect at $5\times10^{-1}\,M$.

Monoamino-dicarboxylic acids at $5\times10^{-3}\,M$ increased the rate of inactivation as the result with glutamate illustrated in Fig. 6. In the case of aspartate, the result was the same.

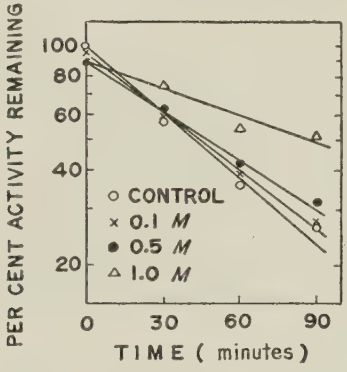

Fig. 5. Influence of glycine. Experimental conditions, *cf.* Fig. 1.

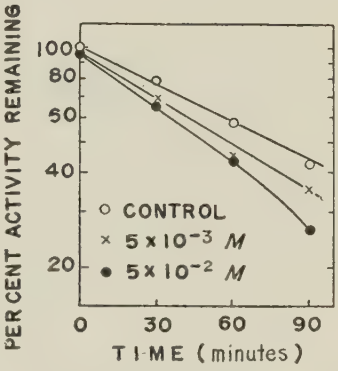

Fig. 6. Influence of glutamic acid. Glutamic acid solution was previously neutralized with NaOH.

Histidine and arginine at the concentration of $1\times10^{-2}\,M$ showed little effect upon the inactivation or rather slightly increased the rate of inactivation (data are omitted).

Methyl- and *n*-propyl-amine were also tested. Methyl amine at $5\times10^{-2}\,M$ and *n*-propylamine at $5\times10^{-3}\,M$ slightly increased the inactivation rate. Propylamine at $5\times10^{-2}\,M$ inhibited the enzyme about 60 per cent under 63°.

DISCUSSION

All of inorganic ions, organic acids, monoamino-dicarboxylic acids and amines tested in this study, increased more or less the rate of heat-inactivation of sweet potato β-amylase. Although these compounds have electric charge of positive or negative, there has been no recognizable specific difference between positive and negative charge, in the experiments. However, the larger ions appeared to have greater instabilizing effects in the case of halogenides and alkaline earthes.

From the facts described above, especially from the fact that the effects

of mono-, di- and tri-basic organic acids depend neither on their molecular weight nor the presence of hydroxyl group, but on the number of carboxylic groups, it seems that the effects of these electrolytes upon the heat-inactivation of β-amylase depend chiefly on their electric charge. It may be suggested, therefore, that on the active group or its vicinity of amylase protein, there arises some strain by electric charge, and the protein molecule may become susceptible to structural change by heat.

It was previously reported (*9*) that sweet potato β-amylase was inactivated by heat following first-order kinetics with respect to time, but that the rate constants of inactivation varied with initial intact enzyme concentration, and the following experimental equation was formulated between rate constant (k) and initial intact enzyme concentration (C_i):

$$k=\alpha C_i^{-\beta}$$

where α and β are constants at the experimental condition. Although the

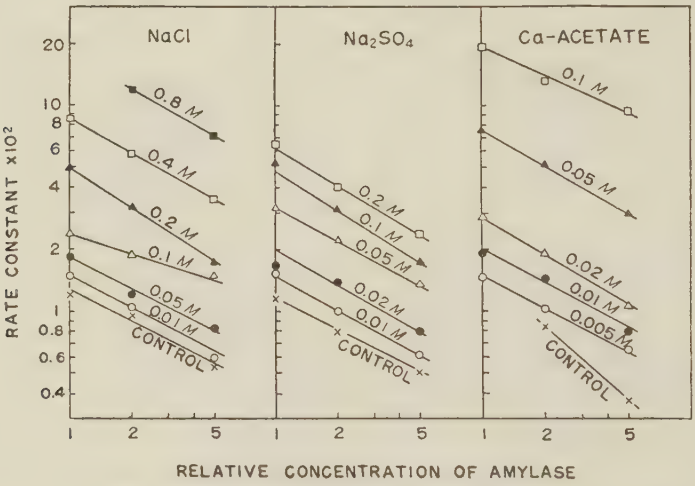

FIG. 7. Log k-log C_i curves of heat-inactivation in the presence of inorganic ions.

theoretical basis of this equation is yet unknown, it may be suggested that the physical state of enzyme protein molecule on which the stability of the protein depends, is a function of total concentration of protein itself.

The term $C_i^{-\beta}$ would concern with the physical state of enzyme protein molecule, and α with the rate of inactivation of enzyme (α is the rate constant at unit concentration of C_i).

Now, the rate constants were plotted against enzyme concentrations at fixed concentration of inorganic ions, in the same manner as in the previous report, from the data in Fig. 2, then it seemed that parallel straight lines were obtained, regardless of the species or concentrations of ions, as illustrated in Fig. 7. In other words, at the different concentrations of ions only α was different, but β constant. This fact may suggest that the presence of

these inorganic ions would concern only with the rate of inactivation, but not with the physical state of enzyme protein molecule.

SUMMARY

The influences of inorganic and organic compounds upon the rate of heat-inactivation of sweet potato β-amylase, were investigated.

Inorganic ions, such as halogenides, cyanide, sulfate phosphate and alkaline earthes, more or less increased the rate of inactivation at $10^{-2}\,M$, but had little effect at $10^{-3}\,M$.

In the presence of organic acids, with the increase in number of carboxylic groups in the acid molecule there was an increase in the inactivation rate. And the effects were proportional to the concentration of carboxylic group.

Most monoamino-monocarboxylic acids and diamino-monocarboxylic acids, of which solubility at pH 5.4 is high enough to test at $10^{-2}\,M$, had little or no effect upon heat-inactivation rate of the β-amylase, except glycine which had slight protective effect at $1\,M$. But monoamino-dicarboxylic acids at $5\times10^{-3}\,M$ increased the rate of inactivation. And meteyl- and *n*-propyl-amine slightly increased the inactivation.

From the facts described above, it is assumed that the heat-inactivation of speet potato β-amylase is stimulated by electric charge (positive or negative) of ionic substances, which is added in the system.

We are deeply indebted to Prof. S. Akabori and K. Okunuki, Faculty of Science, Osaka University, for their encouragements and valuable suggestions.

REFERENCES

(*1*) Taylor, J. F., The Proteins, Ed. by H. Neurath and K. Bailey, Academic Press, New York, Vol. **I**, p. 35 (1953)
(*2*) Schneyer, L. H., *Arch. Biochem. Biophys.*, **39**, 65 (1952)
(*3*) Caldwell, M. L., and Kung, J. T., *J. Am. Chem. Soc.*, **75**, 3132 (1953)
(*4*) Hagihara, B., *Ann. Rept. Sci. Osaka Univ.*, **2**, 35 (1954)
(*5*) Muus, J., Brockett, F.P., and Connelly, C.C., *Arch. Biochem. Biophys.*, **65**, 268 (1956)
(*6*) Phillips, L. L., and Caldwell, M. L., *J. Am. Chem. Soc.*, **73**, 3559 (1951)
(*7*) Okazaki, H., *J. Agr. Chem. Soc. Japan.* (in Japanese), **28**, 51 (1954)
(*8*) Yamamoto, T., *Bull. Agr. Chem. Soc. Japan*, **20**, 188 (1956)
(*9*) Nakayama, S., and Kono, Y., *J. Biochem.*, **44**, 25 (1957)
(*10*) Giri, K. V., *J. Indian Chem. Soc.*, **11**, 339 (1934)
(*11*) Giri, K. V., Nigam. V.N., and Saroja, K., *Naturwissenschaften*, **40**, 484 (1953)

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CRYSTALLINE BACTERIAL PROTEINASE

II. GENERAL PROPERTIES OF CRYSTALLINE PROTEINASE OF BACILLUS SUBTILIS N'

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(Received for publication, October 2, 1957)

In the preceding paper (*1*) an improved method for preparing crystalline proteinase of *Bac. subtilis* was described. Several properties of crystalline proteinase obtained by the previous method (*2*) from a few strains of *Bac. subtilis* were already reported by Hagihara, and there was no marked difference among crystalline proteinase of different strains of *Bac. subtilis* (*2*). Since the crystalline enzyme is most conveniently obtainable from a strain denoted as *B sub. N'*, more detailed investigation has been carried out on the properties of this proteinase obtained by the improved method (*1*), and some results are presented here.

MATERIALS AND METHODS

Crystalline Proteinase of Bac. subtilis—A commercial crystalline proteinase preparation, "Nagarse" prepared in Amagasaki Factory of Nagase & Co., Ltd. by our improved method (*1*) from a culture medium of *B. sub. N'*, was further purified by careful recrystallization, since the preparation contained some of the autodigestion product of the enzyme as impurity. This crystalline preparation is denoted as BPN' in this paper.

*Assay for Casein Digestion Activity***—The activity of casein digestion was determined by the method described in the preceding paper (*1*), which was slightly modified as follows: The determination of the amount of digestion product, which was not precipitated with the precipitation reagent B***(trichloroacetic acid-acetic acid-sodium acetate mixture (*1*)), was carried out by the Folin color reaction in which 5 ml. of 0.55 *M* sodium carbonate followed by 1 ml. of 1/3-diluted Folin-Ciocalteu reagent (*4*) were added to 2 ml. of the filtrate and optical density of the resulting colored solution was measured at 660 mμ after standing at 30° for 30 minutes. The activity unit, μg-tyrosine unit, was defined similarly as in the preceding report (*1*) and measured values were corrected by the standard activity curve shown in Fig. 1, in which the curve obtained by precipitation reagent A (CCl_3COOH) is also shown for comparison. For the assay of the activity at various pH, a buffer system containing 0.033 *M* phosphate and borate was used instead of phosphate

* Nagase & Co., Ltd., Amagasaki Factory, Amagasaki, Japan.

** Detailed procedures were described by Hagihara *et al.* in "Proteolytic Enzyme Research" II, Section 6, 237-254 (1956), Asakura Shoten, Tokyo.

*** Reagent A appeared in the preceding paper [J. Biochem. **45**, 186 (1958)] must be read reagent B.

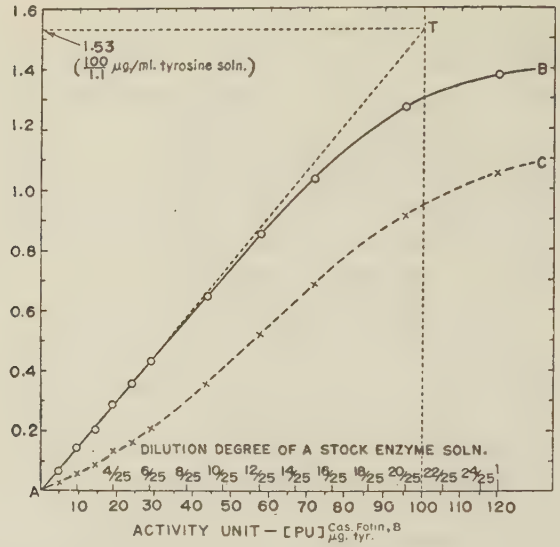

FIG. 1. Standard curve for assaying bacterial proteinase by Casein-Folin method.

Stock enzyme solution; About 80μg./ml. solution of BPN'.

Reaction; 1 ml. of enzyme, 5 ml. of 1.2 per cent casein at pH 7.5, 30° for 10 minutes.

Precipitation reagent; 5 ml., reagent B for curve AB, reagent A for curve AC.

Color reaction; 2 ml. of the filtrate, 5 ml. of 0.55M Na_2CO_3, 1 ml. of 1/3 Folin reagent. Optical density was measured using 66 mμ filter.

Activity unit; 1 unit was defined as the activity which liberated, per minute at an initial reaction, the digestion product not precipitated with the precipitation reagent which gave the same Folin color as 1μg. of tyrosine (see following formula). Tangent line AT was used for the correction of measured value.

Standard tyrosin solution; 20μg/ml. solution was used. The optical density of Folin color of this solution, 0.306, corresponded to 20 unit of activity, and therefore, the value 1.53 correspond to 100 unit, was decided from this value and used for the standard point to graduate the activity unit in the figure.

$$[PU]^{\text{cas. Folin}}_{\mu\text{g. tyr.}} = \frac{\text{Folin color of the reaction filtrate}}{\text{Folin color of 1}\mu\text{g/ml. tyrosin soln.}} \times \frac{\text{Volume of the filtrate}}{\text{Reaction time}}$$

$$= \frac{\text{FOD measured}}{\text{FOD of 1}\mu\text{g./ml. tyr.}} \times \frac{11}{10}$$

$$= \text{FOD measured} \times \frac{1.1}{\text{FOD of 1}\mu\text{g./ml. tyr.}}$$

buffer in the assay at neutral pH, and correction by the standard curve was not carried out.

Assay for Peptide Bond Hydrolysing Activity—One ml. of the enzyme solution was added to 5ml. of 3 per cent casein solution containing 0.02M sodium phosphate-ammonium hydroxide buffer system at various pH, and kept at 30° for 20 minutes. After the reaction, 5 ml. of 40 per cent formol solution of neutral pH was added to the mixture and elec-

trometric titration was carried out with 0.01 *N* sodium hydroxide. As the end point of the titration, pH 8.3 was adopted and the titration values were corrected for the values of blanks which were prepared mixing substarte solution with the formol solution before addition of the enzyme.

Assay for Esterase Activity—Mixture of 1ml. of the enzyme solution and 5ml. of about 1 per cent solution (or suspension) of ester in 0.02 *M* acetic acid-trisaminomethane-ammonium hydroxide buffer system at various pH was allowed to react at 30° for 20 minutes to 2 hours, 5 ml. of 40 per cent formor solution was added, and then titrated with 0.01 *N* sodium hydroxide using a mixture of cresol red and thymol blue as the indicator. Though the above buffer system showed a strong buffering capacity from pH 4.0 to 11.0, the capacity was lost by the addition of formol in the case of titration and a sharp end point was obtained. The titration value was corrected for the blank value before.

Other procedures are described in the following section.

RESULTS AND DISCUSSION

Homogeneity of the Crystalline Preparation—The once recrystallized proteinase of *B. sub. N'* showed only a single peak in electrophoresis performed at pH

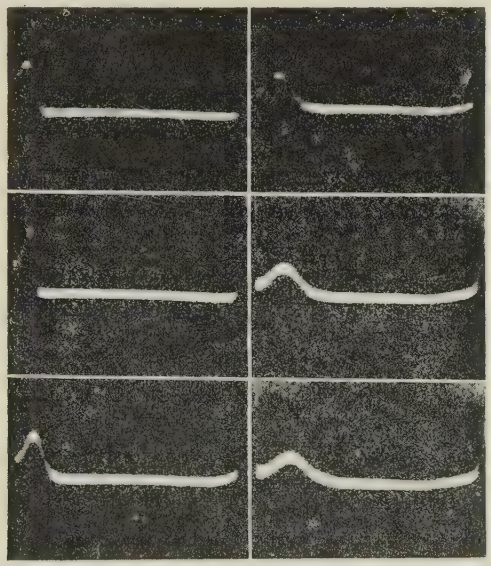

FIG. 2. Ultra-centrifugal diagram of crystalline proteinase of *Bac. subtilis N'*.

Solvent; 0.1 *M* phosphate buffer, pH 7.5. Protein concentration; about 0.8per cent. RPM; 49,500 in Hywe-type Ultracentrifuger Time interval; 15 minutes.

7.0 using a 0.05 *M* sodium phosphate buffer by Hitachi Tiselius apparatus. At pH 6.0, however, another peak correspoding to about 20 per cent of the main component was observed. It was not confirmed whether this peak was due to the presence of an impurity in the preparation or by the enzyme itself modified during the dialysis for 48 hours under this rather dangerous

pH for the enzyme.

The preparation was shown to be a homogeneous substanece by the ultracentrifugal experiment performed by a Dr. H. Inagaki of the Institute of Chemical Research, Kyoto University.

Analytical Results—The elementary composition of the twice recrystallized preparation was as follows: C 49.5, H 7.5, N 15.1, S 1.0, and ash 1.33 per cent. Almost all of metal contained in the above ash was calcium.

From the results of two-dimensional paper chromatography and other qualitative tests, it was almost certain that the enzyme was composed of following amino acids: Aspartic acid, asparagine, glutamic acid, glutamine, glycine, alanine, valine, leucine, isoleucine, serine, threonine, histidine, lysine, arginine, methionine, proline, tyrosine, phenylalanine, and tryptophan.

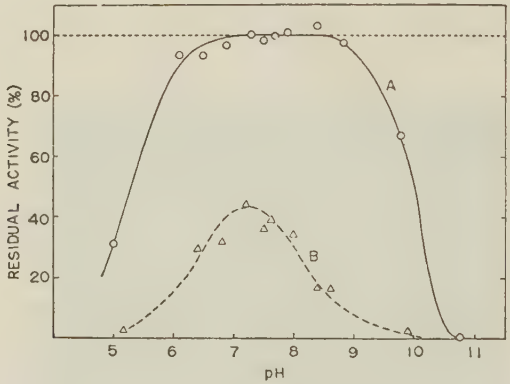

Fig. 3. Relation between stability and pH of BPN′.
Curve A: After incubation of 0.01 per cent BPN′ containing *M*/15 sodium phosphate-borate buffer at 31° for 8 hours.
Curve B: After incubation at 50° for 30 minutes.

As the terminal amino acid residue of the peptide chain of the enzyme protein, only alanine was found by Sanger's method (*5*).

Stability—The enzyme completely dialyzed against distilled water was very unstable at room temperature. The enzyme was highly protected from inactivation in the presence of many salts especially ammonium, calcium, sodium, and cobalt salts even at the concentration of 0.001 *M*. The diluted enzyme solution containing one of the above salts was considerably stable at neutral pH below 40°.

Relation between stability of the enzyme and pH of the solution, which containg sodium ion of about 0.1 *M* as a component of buffer and trace of calcium ion as a component of the enzyme protein, is shown in Fig. 3. In the case of diluted solution, about 0.01 per cent, the enzyme was quite stable during incubation at 31° for 8 hours at the pH range of 6.5–9.0, but

TABLE I

Protecting Action of Bacterial Cell Extract and Salts on Inactivation of BPN′

Time of incubation		0 hr.		4 hr.		6 hr.	
Shaking	Addition	Act.*	Non-prot.**	Act.*	Non-prot.**	Act.*	Non-prot.**
−	−	45.1	0.073	26.5	0.138	33.0	0.173
+	−			21.0	0.223	14.5	0.258
−	−	74.0	0.113	57.0	0.160	46.0	−
−	cell ext.	74.0	—	66.0	—	61.0	—
−	0.1*M* NH_4-PO_4 (pH 7)	,,	0.120	61.5	0.145	52.5	—
−	0.1*M* $(NH_4)_2SO_4$	,,	—	63.0	0.150	51·0	—
+	−	,,	—	36.5	0.243	19.0	—
+	cell ext.	,,	—	54.5	—	40.5	—
+	0.1*M* NH_4-PO_4 (pH 7)	,,	—	50.5	—	30.5	—
+	0.1*M* $(NH_4)_2SO_4$	,,	—	48.0	—	32.0	—

Enzyme concentration; about 0.2–0.5 per cent. pH; 7.0 using 0.05 *M* sodium phosphate buffer. Temperature; 35°, Shaking; 110 per minute.

* Remaining activity was expressed by the tyrosine unit (see Fig. 1.) of 30 fold diluted enzyme.

** Non-protein substance produced by autodigestion of the enzyme protein was measured by the Folin color reaction after the addition of trichloroacetic acid at the final concentration of 0.4 *M* and filtration.

fairly unstable at 50°, even at the most stable pH of 7.0–7.5.

The enzyme was gradually inactivated at 35° and the inactivation was much increased in a higher concentration of the enzyme (see following report (*6*)). This implies the fact that the above inactivation of BPN′ is caused by autodigestion of the enzyme in a way similar to inactivation of trypsin at neutral pH, though the inactivation of BPN′ is much smaller than that of trypsin. This is easily supposed from the fact that non-protein substance increased during the course of inactivation as shown in Table I. The above inactivation was further promoted by shaking the euzyme solution. It is shown in Table I that the inactivation was strongly protected by the addition of the extract of bacterial cells and ammonium salt, both in settling and shaking experiment of the neutral enzyme solution containing phosphate buffer. The protective effect of ammonium salt was more marked in the case of shaking than settling. Other metal salts such as calcium chloride and sodium chloride were slightly or not effective in the above experiment, though they were effective when they were added to the enzyme solution containing almost no salts (*2*).

Activity and pH—Relation between pH and activity of BPN′ was measured by the Casein-Folin reagent method and Casein-formol electric titration method are shown in Fig. 4. As shown in the figure, activity of the

peptide bond hydrolysis measured by the titration method showed a rather sharp optimum pH at pH 7.5–8.0 as in the case of trypsin and chymotrypsin. However, casein digestion activity, *i. e.* non-protein substance-producing activity, measured by the Folin method was optimum at pH 10–10.5 and the activity was considerably high through a wide range of pH from 7.0 to 11.0. Practically the same result was obtained when the activity was measured by the Casein-275 mμ method. It was supposed from the above results that casein was digested to the state which could not be precipitated with the protein precipitation reagent in nearly the range of pH 7.0–9.0 and somewhat higher at pH 10–10.5. Nevertheless, the hydrolysis of peptide bonds in casein molecule was strongly catalyzed only at pH 7.5–8.0. It is not

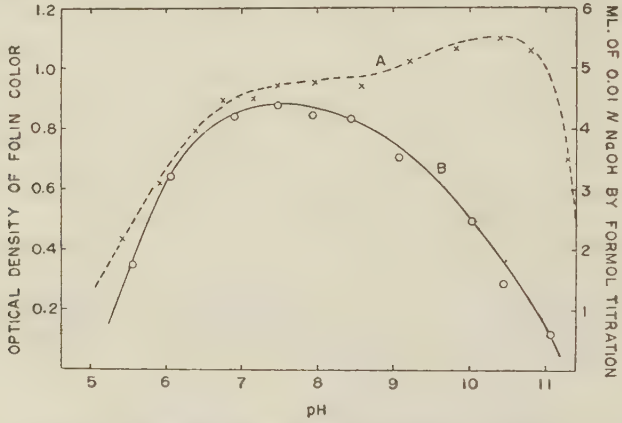

Fig. 4. Relation between pH and activity of BPN′ (*1*).

Curve A: Casein-Folin color reaction method using 0.005 per cent BPN′, about 0.3*M* phosphate-borate buffer and modified protein precipitation reagent B containing 0.44 *M* acetic acid.

Curve B: Casein-formol titration method using about 0.025 per cent BPN′ 0.02 *M* sodium phosphate-ammonium hydroxide buffer.

impossible to consider that the precipitation of the undigested casein with the precipitation reagent is distribution in the solution at higher pH. However, buffering capacity of the precipitation reagent was much stronger than that of the reaction solution and final pH of the mixture of the reaction solution and the precipitation reagent was almost the same in the reaction solution at various pH. Therefore, the above probability is thought to be very small.

Esterase Activity—It was reported by Güntelberg and Ottesen that a crystalline proteinase, "subtilisin", obtained by them from a strain of *Bac. subtilis*, was able to hydrolyze methyl butyrate (*7*). Graae also investigated the action of bacterial proteinase upon several esters (*8*). The present proteinase, BPN′, also hydrolyzed several esters such as methyl butyrate, ethyl valerate, and ethyl isovalerate in the state of solution or suspension in water, but ethyl acetate was hardly hydrolyzed. Since methyl

butyrate was most easily hydrolyzed by the enzym among the above susceptible esters and also it is soluble in water to a sufficient extent, the rete of hydrolysis at various pH was compared with the hydrolysis of gelatin which is soluble in a wider range of pH than casein. As shown in Fig. 5, optimum pH of the ester hydrolysis was at pH 8.5–9.5, while that of gelatin was 7.5–8.0, similar to casein. This difference is thought to be due to the

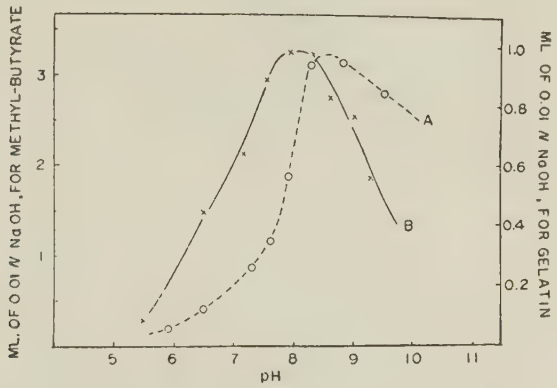

FIG. 5. Relation between pH and activity of BPN′ (*2*).

Curve A: Esterase activity.
Reaction: 1 per cent methyl bvtyrate in the reaction mixture, at 30° for 2 hours.

Curve B: Proteolytic activity.
Reaction: 2.5 per cent gelatin in the reaction mixture, at 30 for 2 hours.

Both reactions were carried out using the same concentration of enzyme and same buffer solution (acetic acid-trisaminomethaneammonium hydroxide system).

fact that ester bonds are weakened at higher pH. The rate of hydrolysis of the ester at its optimum pH was about 8 times smaller as that of gelatin.

SUMMARY

Crystalline proteinase of *Bac. subtilis* was shown to be homogeneous by ultracentrifugal analysis. Diluted solution of it was considerably stable at pH 6–9 below 40° but hardly at 50°, at which it was most stable at pH 7.0–7.5. Concentrated enzyme solution was somewhat unstable even at neutral pH even below 40° owing to its autodigestion. This inactivation was much promoted by shaking, and protected by the bacterial cell extract and ammonium salt. Optimum pH of the enzyme measured by formol titration method was about 8.0 and that by casein-Folin method was about 10.0, though by the latter method it is fairly active in a very wide range of pH from 7.0 to 11.0. Fatty acid esters such as methyl butyrate, ethyl valerate, and isovalerate, were susceptible to the enzyme, and optimum pH of the methyl butyrate hydrolysis was about 9.0.

REFERENCES

(*1*) Hagihara, B., Matsubara, H., Nakai, M., and Okunuki, K., *J. Biochem.*, **45**, 185 (1958)
(*2*) Hagihara, B., *Ann. Rep. Sci. Works. Osaka Univ.*, **2**, 35 (1954)
(*3*) Kunitz, M., and Northrop, J.H., *J. Gen., Physiol.*, **18**, 443 (1935)
(*4*) Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927)
(*5*) Sanger, F., *Biochem. J.*, **39**, 507 (1945)
(*6*) Hagihara, B., Nakai, M., Matsubara, H., Komaki, T., Yonetani, T., and Okunuki, K., *J. Biochem.*, in press.
(*7*) Güntelberg, A.V., and Ottesen, M., *Compt. rend. Lab. Carlsberg, Ser. chim.*, **29**, 36 (1954)
(*8*) Graae, J., *Acta Chem. Scand.*, **8**, 356 (1954)

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ZONE ELECTROPHORESIS STUDIES OF HABU (CROTALIDAE) VENOM

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In 1938, Slotta and Fraenkel-Conrat (*1*) crystallized "Crotoxin" from *Crotalus t. terrificus* which possessed both toxic and enzymatic properties. Ghosh and De (*2*), however, suggested that it might contain at least two components, real toxin and phospholipase A. Recently Michl (*3*) investigated by zone electrophoresis the relation between enzymatic activities and toxicity on *Bothrops* venom but failed to separate phospholipase A from toxic activity. Bussard and Côté (*4*) and Neumann and Habermann (*5*) succeeded in separating toxin from phospholipase A (on *Naja naja* (*4*), on *Clotalus t, terrificus* (*5*)) by electrophoresis and chromatography, respectively. Grassmann and Hannig (*6*) have demonstrated that paper electrophoresis of some venoms such as *Naja nigricollis*, *Bothrops jararaca* or *Black Cobra* gave two hemolytic fractions. In all the above experiments except in that of Neumann *et al.* enzymatic activies and toxicity were observed qualitatively.

In this report the present author deals with the zone electrophoretic separation with potato starch as the supporting medium of the venom of *Trimeresurus flavoviridis* (*Habu*), a *Crotalidae*. The main purpose is to separate the toxicity from phosphoripase A and other enzymatic activities on the basis of as precisely quantitative an analysis as possible. Evidences were obtained to show that there are at least two hemolytic activities in this venom and both two were separated from toxicity.

MATERIALS AND METHODS

Snake Venom—The venom used was a dried pool collected in 1948 from species of *Trimeresurus flavoviridis* (Hallowell)*. The test venom solution was prepared in physiological saline immediately before use.

Substrate—Calcium-bis-*p*-nitrophenyl phosphate was prepared from tris-*p*-nitrophenyl phosphate according to the method of Yoshida (*7*). The tris-*p*-nitrophenyl phosphate was prepared according to Rapp (*8*).

Adenosine-5′-phosphate was purchased from L. Light & Co., Ltd.

* We are indebted to Dr. K. Nakamura, Vice-director of National Institute of Health, Tokyo, for the kind supply of the venom of *Trimeresurus flavoviridis* (Hollowell).

Toxicity—Venom toxicities were tested by intravenous injection of serial 1.5-fold dilutions of each fraction into mice weighing from 15 g. to 17 g. in the amount of 0.2 ml. Four animals were injected per dilution. The range of dilutions inoculated was from the lowest with which 100 per cent mortality was obtained to the highest causing no mortality. The LD_{50} (50 per cent lethal dose) was calculated by the Reed-Muench method.

The MLD minimum lethal dose) and LD_{50} of the crude venom used were approximately 100 and 60 μg, respectively, for mice weighing from 15 to 17 g. Although in all the data presented in the present paper the toxicity is expressed by the intravenous lethal doses, data available at hand indicate that the experiments can as well be carried out by intraperitoneal injection of mice.

Estimation of Protein—The protein content of the venom solution was expressed as the optical density at 280 mμ using a Beckman DU spectrophotometer by 1 cm. cell, after the appropriate dilution with physiological saline. Physiological saline was used as a blank. Under these conditions a solution containing 1 mg. protein 1ml. in crude venom was found to have an optical density of *ca.* 1.5.

Phospholipase A Activity –Phospholipase A activity was followed indirectly as the hemolysing activity of the incubation mixture. All reagents were diluted with veronal-buffered saline (pH 7.0) recommended by Stein *et al.* (*9*). A 1.5-fold dilution series of a sample was prepared in the volume of 0.25 ml. To each tube were added 0.25 ml. of 0.05 per cent lecithin suspension, 0.25 ml. of 2 per cent sheep erythrocyte suspension and 0.5 ml. of veronal-buffered saline (pH 7.0). Incubation for one hour at 37° was followed by placing the tubes overnight in the ice box at about 4°. After this period, the hemolysin titer was expressed as the highest dilution of the venom solution showing complete hemolysis.

Phosphodiesterase Activity—Determination of phosphodiesterase activity was performed on Ca-bis-*p*-nitrophenyl phosphate by the method of Shinsheimer and Koerner (*10*) with the modification of shortening the incubation time.

5'-Nucleotidase Activity—Determination of 5'-nucleotidase activity was carried out on adenosine-5'-phosphate by the method of Private de Garilhe and Laskowski (*11*) using Allen's method (*12*) for determination of inorganic phosphate.

Zone Electrophoresis—The zone electrophoresis was carried out with potato starch as the supporting medium in an apparatus similar to that of Kunkel and Slater (*13*). The commercial potato starch was washed with a large volume of water until substances showing the spectra at 280 mμ in washings were completely removed, being followed finally by several times of washings with the buffer solution to be employd. This starch preparation was used as the supporting medium. A portion of this preparation was dried and used to solidify the input material.

The run was made at about 4° in a pH 9.2, 0.11 ionic strength borax-NaOH buffer. The starting sample, amounting about 450 mg. of crude venom in 4.5 ml. of physiological saline was mixed with dried starch and indroduced into a 1 cm. slit cut crosswise in a 50× 5×1.5 cm. block of the starch. The starting trench was set at a point 5 cm. apart from the middle point of block toward anode. A current of about 470 volts and 18 mA was applied for 12 hours. The starch block was then cut crosswise into sections 1 cm. wide, each of which was extracted with 10 ml. of physiological saline. Estimation of protein, toxicity and enzymatic activities were made on appropriate aliquots of the extracts.

RESULTS

Results obtained with the crude venom solution when this was subjected to electrophoresis in borax-NaOH buffer at pH 9.2, μ=0.11 for 12

hours at 2.4 mA/cm^2., are presented in Figs. 1-A, 1-B and 1-C. Optical density at 280 mμ for protein content and recovery percentage of toxicity and of enzyme activities of eluate are plotted as a function of the distance from the starting trench.

The pattern of distribution of toxicity and enzyme activities showed that at least two hemolytic activies exist and both of them were separated from toxicity (see Fig. 1-A), and that phosphodiesterase and 5′-nucleotidase activities were also separated from toxicity (see Fig. 1-B and -C). The total recoveries calculated from the starting material of protein, toxicity and enzyme activities were as follows; protein 102 per cent toxicity 51 per cent, hemolytic activities more than 73 per cent, phosphodiesterase activity 99 per cent and 5′-nucleotidase activity 78 per cent.

The specific activities of enzymes and of toxin per unit amount of protein in eluates are expressed in Fig. 2 as the ratio to those of the crude venom.

DISCUSSION

It was found that at pH 9.2 the venom of *Trimeresurus flavoviridis* gave two electrophoretically separable fractions both of which have hemolytic activity, while Slotta (*14*) showed that at pH 5.18 the same venom gave electrophoretically a single fraction with hemolytic activity. Grassmann and Hannig (*6*) have qualitatively demonstrated that some venoms such as *Naja nigricollis*, *Bothrops jararaca* or *Black Cobra* gave two hemolytic fractions by paper electrophoresis at pH 8.6. Thus it is very interesting to know whether or not all snake venoms have more than two hemolytic activities, and whether or not these activities are due to phospholipase A action. At present we do not know to what extent these two hemolytic activities are different from each other in principle. These problems remained to be ascertained.

It was also shown from Fig. 1-A that both of these two hemolysins in this venom were separated from toxicity. Recently Bussard and Côté (*4*) reported the zone electrophoretic separation of phospholipase A from neurotoxic activity on *Naja naja*. Neumann and Habermannn (*5*) also suceeded in separating by chromatographic technique phospholipase A from toxic activity on *Clotalus t. terrificus*. Therefore in view of our results and of these reports, evidences have been presented to show that hemolysin is not identical with the toxin in the venom of *Colubridae* nor of *Viperidae*.

As seen in Fig. 1-A in addition to the main toxic fraction another lower toxic peak is present in this venom to give the yield of about 4 per cent of total original toxicity. The specific activity of these two toxic peaks were in the same order (see Fig. 2). Therefore it can be suggested that at least two toxic substances are present in this venom.

According to Taborda *et al.* (*15*) the average phosphodiesterase content in venoms of various species showed a significant parallelism with their

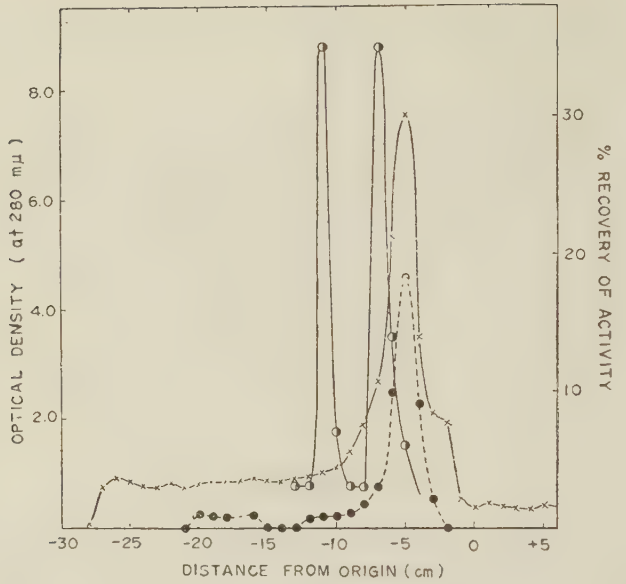

Fig. 1-A. The starch zone electrophoretic pattern of crude venom showing the distribution of protein (—×—), toxicities (- -●- -) and hemolytic activities (—◑—), at pH 9.2 (borax-NaOH buffer μ=0.11) for 12 hours at 2.4 mA/cm^2.

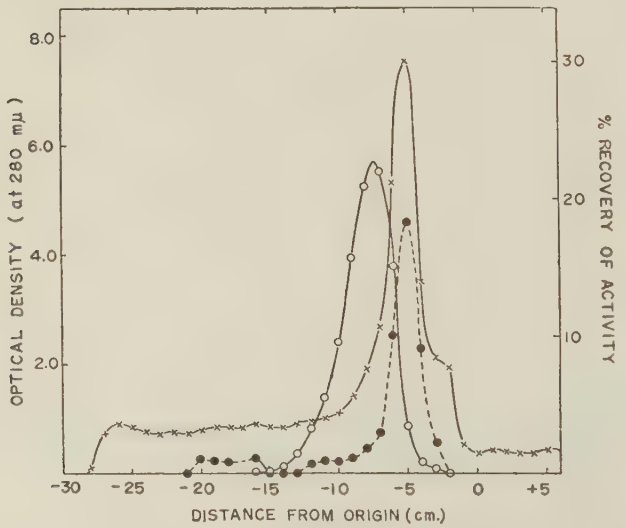

Fig. 1-B. Distribution of protein (—×—), toxicities (- -●- -) and phosphodiesterase activities (—○—) in the same electrophoretic run as in Fig. 1-A.

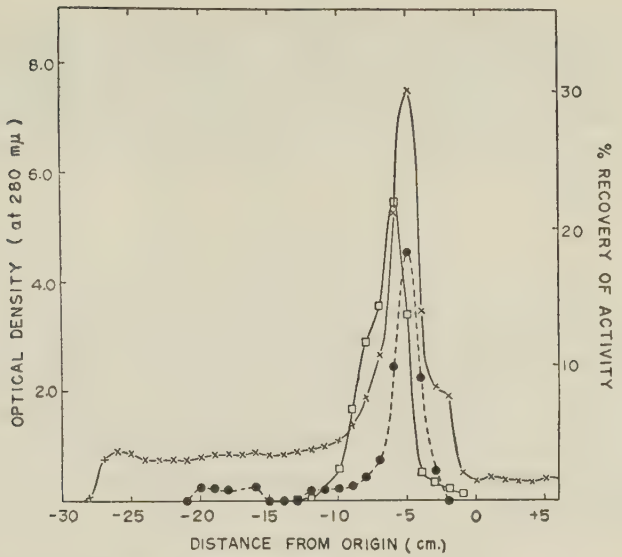

FIG. 1-C. Distribution of protein (—×—), toxicities (- -●- -) and 5′-nucleotidase activities (—□—) in the same electrophoretic run as in FIG. 1-A.

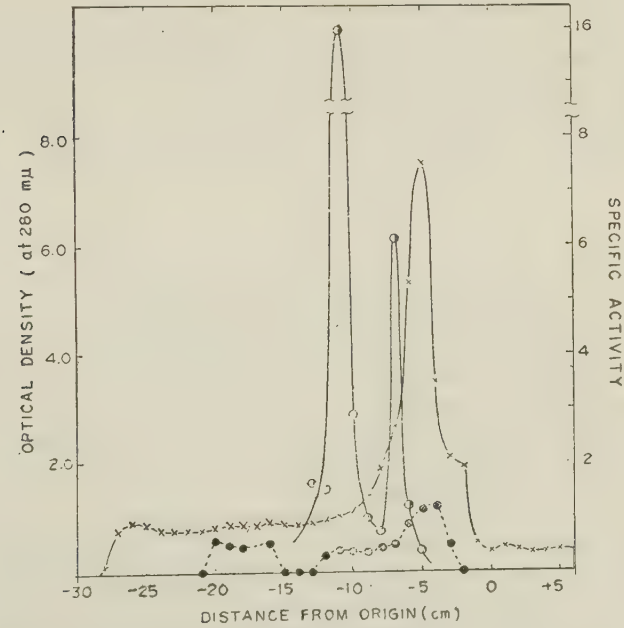

FIG. 2. The specific activities per unit amount of protein of toxin (- -●- -) and of hemolytic substances (—◑—) in the same electrophoretic run as in Fig. 1-A. Distribution of protein (—×—) is also shown.

toxicity. Contrary to their suggestion, Fig. 1-B indicated that there was no correlation of phosphodiesterase activity with toxicity. Zeller (*16*) considered that 5′-nucleotidase and other phosphomonoesterases together with ATPase are probably responsible for the production of fatal shock. But it was shown from Fig. 1-C that at least 5′-nucleotidase was separated from the venom toxicity.

SUMMARY

1. The venom of *Trimeresurus flavoviridis* (Habu), a *Crotalidae*, was fractionated by zone electrophoresis with potato starch as the supporting medium. Several enzymatic activities and toxicity were determined quantitatively.

2. It was shown that at least two hemolytic activities were present in this venom and both of them were separated from toxicity.

3. Phosphodiesterase and 5′-nucleotidase activities were also separated from the venom toxicity.

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REFERENCES

(*1*) Slotta, K. H., and Fraenkel-Conrat, H. L., *Ber. dtsch. Chem. Ges.*, **71**, 1076 (1938)
(*2*) Ghosh, B. N., and De, S. S., *Nature*, **143**, 380 (1939)
(*3*) Michl, H., *Naturwiss.*, **41**, 403 (1954); Michl, H., *Monatsh. Chem.*, **85**, 1240 (1954)
(*4*) Bussard, A., and Côté, R., *Compt. rend.*, **239**, 915 (1954)
(*5*) Neumann, W. P., *Naturwiss.*, **42**, 370 (1955); Neumann, W. P., and Habermann, E., *Biochem. Z.*, **327**, 170 (1955)
(*6*) Grassmann, W., and Hannig, K., *Z. physiol. Chem.*, **296**, 30 (1954)
(*7*) Yoshida, S., *J. Biochem.*, **34**, 23 (1941)
(*8*) Rapp, M., *Ann.*, **224**, 156 (1884)
(*9*) Stein, G. J., and Ngu, D. V., *J. Immunol.*, **65**, 17 (1950)
(*10*) Shinsheimer, R. L., and Koerner, J. F., *J. Biol. Chem.*, **198**, 293 (1952)
(*11*) Privat de Garilhe, M., and Laskowski, M., *Biochim. Biophys. Acta.*, **18**, 370 (1955)
(*12*) Allen, R. J. L., *Biochem. J.*, **34**, 858 (1940)
(*13*) Glick, D., Methods of Biochemical Analysis, Interscience Publishers, Inc., New York, **1**, p. 141 (1954); Kunkel, H.G., and Slater, R.J., *Proc. Soc. Exptl. Biol. Med.*, **80**, 42 (1952)
(*14*) Slotta, K., and Borchert, P., *Mem. Inst. Butantan.*, **26**, 297 (1954)

15) Taborda, A. R., Taborda, L. C., Williams, J. N., Jr., and Elvehjem, C.A., *J. Biol. Chem.*, **194**, 227 (1952); Chargaff, E., and Davidson, J. N., The Nucleic Acids, Academic Press Inc., Publishers, New York, **1**, p. 555 (1955)

(*16*) Summer, J. B., and Myrbäck, K., The Enzymes, Academic Press Inc., Publishers, New York, **1**, Part 2, p. 986 (1951)

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TERMINAL OXIDATION SYSTEM IN BACTERIA

II. SOME PHYSICAL AND PHYSIOLOGICAL PROPERTIES OF PURIFIED CYTOCHROMES OF PSEUDOMONAS AERUGINOSA*

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In the previous paper (*1*), a simple procedure was described by which three kinds of cytochromes and a blue protein were extracted in water-soluble state from *Pseudomonas aeruginosa*, and methods given by which they could be fairly purified. Several cytochromes from bacterial sources have already been highly purified; Tissierés (*2*) purified cytochrome c_4 from *Azotobacter vinelandii*, Kamen and Takeda (*3*) purified cytochrome c from *Pseudomonas aeruginosa*, and Postgate (*4*), and Ishimoto, Yagi and Shiraki (*5*) purified cytochrome c_3 from *Desulfovibrio desulfuricans*. There has however been no attempt to compare the physical and physiological properties of various cytochromes purified from one bacterial sources. But it has been shown that one bacterial strain's cytochrome may differ from that of another strain even if it has similar spectrophotometrical properties.

In this paper, three kinds of cytochromes (*Pseudomonas* cytochrome$_{551}$, cytochrome$_{554}$, and cytochrome$_{GB}$)** and a coloured protein (*Pseudomonas* blue protein) purified from *Pseudomonas aeruginosa* are compared physically and physiologically and evidences given that cytochrome$_{GB}$ is a cytochrome oxidase.

MATERIALS AND METHODS

Pseudomonas aeruginosa was cultured, and cytochrome$_{551}$, cytochrome$_{554}$, cytochrome$_{GB}$ and blue protein were extracted and purified as reported previously (*1*).

DPNH-cytochrome c reductase was prepared from pig heart muscle by the method of Mahler, Sarker, Vernon and Alberty (*6*).

TPNH-cytochrom c reductase was prepared from baker's yeast by the method Haas, *et al.* (*7*, *8*). The preparation still contained cytochrome b_2 as judged by its absorption spectrum.

Yeast lactic dehydrogenase was prepared from baker's yeast by the method of Yamanaka, Horio and Okunuki (*9*).

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* This work was attempted and directed by K. Okunuki, and co-operated by T. Higashi and H. Matsubara.

** In this paper, the pigments of *Pseudomonas aeruginosa* are donoted by an abridgement of the source material, or by the letter "P".

Crystalline yeast and bovine cytochrome c's were prepared from baker's yeast and bovine heart muscle as described previously (*10*, *11*, *12*).

Bovine cytochrome c oxidase was prepared in a water-soluble form using sodium cholate according to a modified method (*13*) of Smith and Stotz (*14*).

Zone-electrophoresis was done on starch in a 0.1 *M* phosphate buffer (pH 8.0) by the standard method.

Oxidation reduction potential were measured in a phosphate buffer (pH 7.0) using *o*-chlorophenol-2,6-dichlorophenol indophenol and anthraquinone-2-sulfonate as the oxidizing and reducing agents.

Oxygen consumption was measured with a Warburg manometer.

Spectrophotometric analyses were made at room temperature with a Shimadzu photoelectric spectrophotometer, type QB-50, in 1 cm. cuvettes. For anaerobic measurements, Thunberg type cuvettes were used.

RESULTS

(*1*) *Properties of-P-Cytochrome*$_{551}$—As shown in the previous report (*1*), cytochrome$_{551}$ has a slightly but distinctly different spectrum from yeast or bovine cytochrome c. It is the most easily extracted and purified of the *Pseudomonas* cytochromes. Preparations of cytochrome$_{551}$, purified by aluminum oxide chromatography (*1*), can be further purified by zone-electrophoresis and acetone fractionation. After these purification procedures, the preparation has the absorption spectrum shown in Fig. 1. The redox-potential of

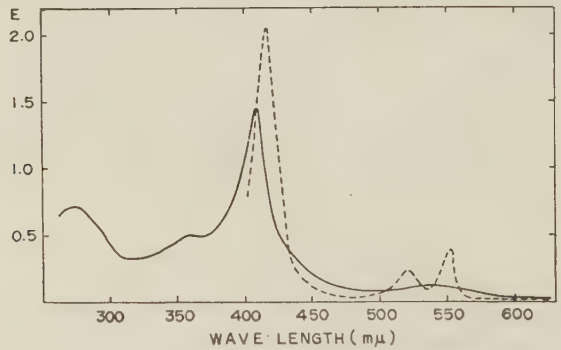


Fig. 1. Absorption spectra of *Pseudomonas* cytochrome$_{551}$. Solid line, in oxidized form; broken line, in reduced form.

cytochrome$_{551}$ is +250 mV. The bovine cytochrome c which has been shown to be most native as judged by cation exchanger chromatography and bacterial proteinase digestion (*12*), has a redox-potential of +243 mV. Of the *Pseudomonas* cytochromes, cytochrome$_{551}$ has a redox-potential nearest to that of bovine cytochrome c.

DPNH-cytochrome c reductase (*6*) prepared from pig heart muscle, and yeast lactic dehydrogenase (*9*) prepared from baker's yeast can both catalyze the electron transport between their substrates and cytochrome$_{551}$, as shown

in Fig. 2. TPNH-cytochrome c reductase can also catalyze the electron transport. Like native cytochrome c, cytochrome$_{551}$ shows scarcely autoxidizability in its reduced form, and when ascorbic acid is added to oxidized cytochrome$_{551}$ as a hydrogen donor, oxygen is not consumed, though it is reduced in the acid medium.

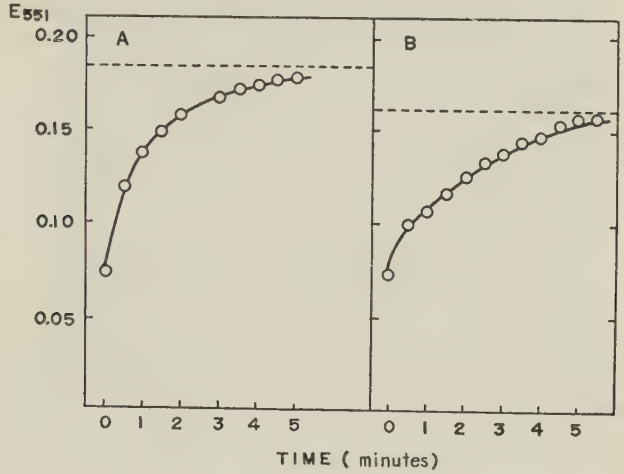


FIG. 2. Reductions of *Pseudomonas* cytochrome$_{551}$ by pig heart-DPNH-cytochrome c reductase, and by baker's yeast lactic dehydro genase. A: By DPNH-cytochrome c reductase prepared from pig heart muscle. Reaction mixture; 1.0 ml. of distilled water, 1.0 ml. of 0.2 *M* phosphate buffer (pH 8.5), 0.5 ml. of cytochrome$_{551}$, 0.2 ml. of DPNH (2 mg./ml.), and 0.1 ml. of the enzyme solution. B: By baker's yeast lactic dehydrogenase. Reaction mixture; 1.5 ml. of distilled water, 0.5 ml. of 0.2 *M* phosphate buffer (pH 6.0), 0.5 ml. of cytochrome$_{551}$, 0.4 ml. of 0.1 *M* sodium lactate and 0.1 ml. of the enzyme solution.

Reactions were started by addition of the enzyme solution. Broken lines showed the extinctions after addition of a trace of sodium dithionite to the reaction mixture.

(2) *Properties of P-Cytochrome*$_{554}$—As described in the previous report (*1*), cytochrome$_{554}$ has an α-band absorption maximum at 554 mμ and is precipitated by a higher concentration of ammonium sulfate than cytochrome$_{551}$. Cytochrome$_{554}$ has a redoxpotential of +225 mV. and can be reduced easily by yeast lactic dehydrogenase, and DPNH-cytochrome c reductase, as shown in Fig. 3. In the case of the reductase, cytochrome$_{554}$ appears to be a somewhat better electron acceptor than cytochrome$_{551}$, and yeast and bovine cytochrome c's. Like cytochrome$_{551}$, cytochrome$_{554}$ is not appreciably autoxidizable in its reduced form.

(3) *Properties of P-Blue Protein*—The purified protein has a brilliant blue colour and is tentatively named as *Pseudomonas* blue protein. Like other cytochromes, this protein can be oxidized and reduced repeatedly, losing its

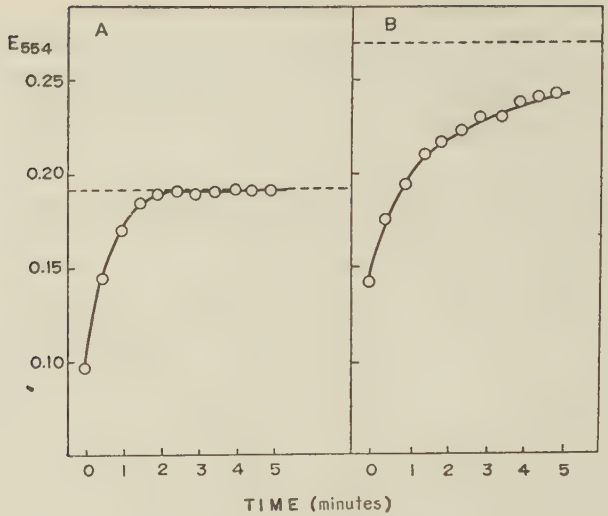


Fig. 3. Reductions of *Pseudomonas* cytochrome$_{554}$ by pig heart-DPNH-cytochrome c reductase, and by baker's yeast lactic dehydrogenase. A: By DPNH-cytochrome c reductase prepared from pig heart muscle. Reaction mixture as for Fig. 2-A, except cytochrome$_{554}$ was used in place of cytochrome$_{551}$. B: By bake'r yeast lactic dehydrogenasse. Reaction mixture as for Fig. 2-B, except cytochrome$_{554}$ was used in place of cytochrome$_{551}$. Broken lines showed the extinctions after addition of a trace of sodium dithionite to the reaction mixture.

colour in reduced form and regaining it on reoxidation. As shown in Fig. 4, blue protein is easily reduced, losing its colour in the presence of lactate and yeast lactic dehydrogenase.

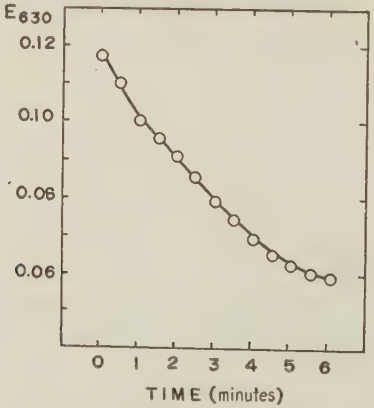


Fig. 4. Reduction of *Pseudomonas* blue protein by baker's yeast lactic dehydrogenase. Reaction mixture as for Fig. 2-B, except use of *Pseudomonas* blue protein instead of cytochrom$_{551}$.

Blue protein was further purified by use of zone-electrophoresis on starch, and crystallized from ammonium sulfate solution in the needle-like form shown in Fig. 5. At this degree of purity, it shows the absorption spectra shown in Fig. 6. The crystals contain approximately one copper atom per each 30,000 g. of protein, and have a redox-potential of +300 mV. It is not appreciably autoxidized in its reduced form.

FIG. 5. Crystals of *Pseudomonas* blue protein.
Once recrystallized sample. Photographed, ×1600.

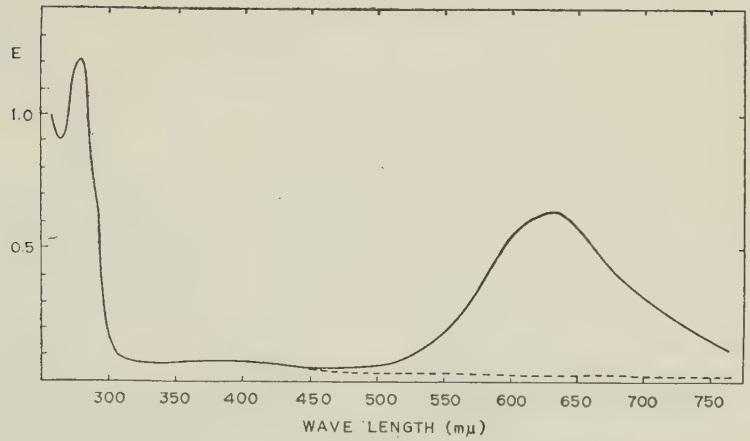


FIG. 6. Absorption spectra of *Pseudomonas* blue protein. Once recrystallized sample was used. Solid line, in oxidized form; broken line, in reduced form.

Electron transport between reduced cytochrome$_{554}$ and oxidized cytochrome$_{551}$, and between reduced cytochrome$_{551}$ and oxidized blue protein, according to the order of their redox-potentials, can be seen with a hand-spectroscope.

(*4*) *Properties of P-Cytochrome*$_{GB}$—As has been shown in the previous report (*1*), cytochrome$_{GB}$ has a complex absorption spectra, suggesting a complex

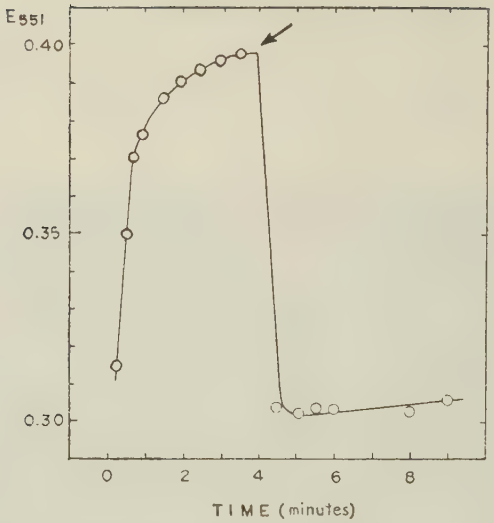


FIG. 7. Reduction of *Pseudomonas* cytochrome$_{GB}$ by baker's yeast lactic dehydrogenase. Reaction mixture; 1.0 ml. of 0.2 *M* phosphate buffer (pH 5.9), 1.5 ml. of cytochrome$_{GB}$, 0.1 ml. of 0.1 *M* sodium lactate and 0.05 ml. of baker's yeast lactic dehydrogenase. The reaction was first carried out under nitrogen, using a Thunberg type cuvette. At the time indicated by an arrow, the nitrogen gas in the cuvette was replaced by air.

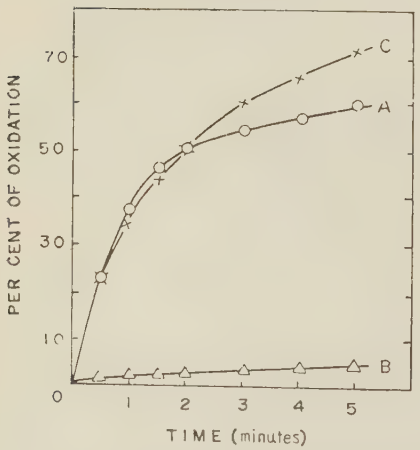


FIG. 8. Oxidations of *Pseudomonas* cytochrome$_{551}$, cytochrome$_{554}$, and blue protein by *Pseudomonas* cytochrome$_{GB}$. Reaction mixture; 1.5 ml. of 0.2 *M* phosphate buffer (pH 5.8), 1.0 ml. of the reduced cytochrome$_{551}$, cytochrome$_{554}$, or blue protein and 0.1 ml. of cytochrome$_{GB}$ (A), +cytochrome$_{551}$; (B), +cytochrome$_{554}$; (C), +blue protein.

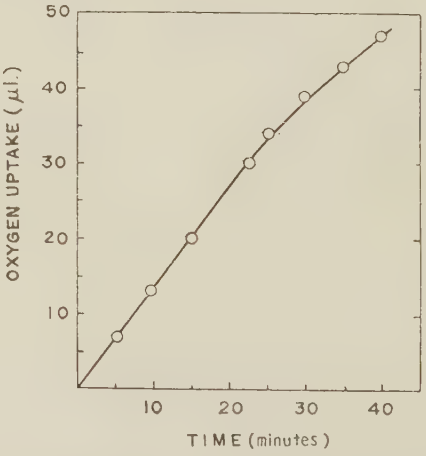


FIG. 9. Oxygen consumption by "Yeast lactic dehydrogenase — *Pseudomonas* cytochrome$_{GB}$" system. Oxygen consumption measured at 30° using a Warburg manometer. Reaction mixture; 0.4 ml. of 0.2 *M* phosphate buffer (pH 5.8), 1.0 ml. of cytochrome$_{GB}$, 0.1 ml. of yeast lactic dehydrogenase, 0.2 ml. of 0.1 *M* sodium lactate, and distilled water to make total volume to 2.0 ml. The reaction was started by adding the dehydrogenase from the side chamber of the vessel.

nature; it shows absorption maxima at 620–630 and 409 mμ in oxidized form, and at 620–630, 551–548, 521–523 and 418 mμ in reduced form. Since these absorption maxima are fairly similar to those of cytochrome$_{551}$, cytochrome$_{554}$ and blue protein, it might be suggested that the preparation of cytochrome$_{GB}$ is contaminated with these substances. However, repeated examinations have shown that cytochrome$_{GB}$ has properties quite different from those of other cytochromes. These include its behaviours on cation exchanger and on aluminum oxide, and the concentration of ammonium sulfate required to precipitate it (*1*).

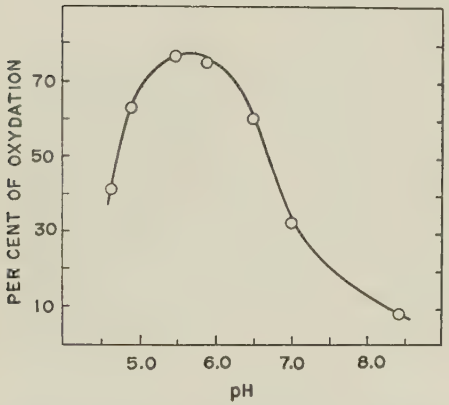


FIG. 10. Influence of pH on the oxidation of *Pseudomonas* cytochrome$_{551}$ by *Pseudomonas* cytochrome$_{GB}$. Reaction mixture; 1.5 ml. of 0.2 *M* phosphate buffer 1.0 ml. of the reduced cytochrome$_{551}$ and 0.1 ml. of cytochrome$_{BG}$. "Percentage of oxidation" was calculated from the following equation:

$$\frac{E^0-E_t}{E_0-E}\times 100$$

E_0; Initial extinction at 551 mμ.
E_t; Extinction at 551 mμ after 2 minutes' reaction.
E; Extinction at 551 mμ when cytochrome$_{551}$ was completely oxidized by addition of a trace of ferricyanide.

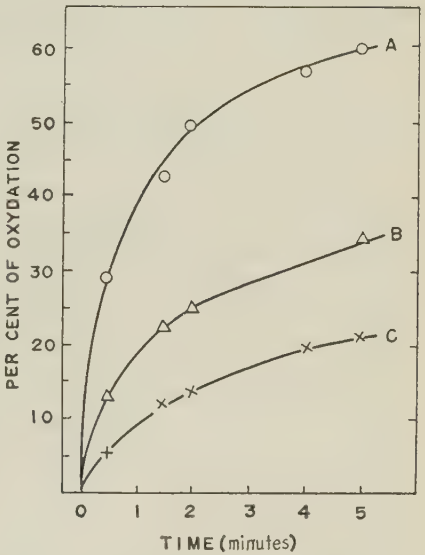


FIG. 11. Inhibition by cyanide of the oxidation of reduced *Pseudomonas* cytochrome$_{551}$ by *Pseudomonas* cytochrome$_{GB}$. Reaction mixture; 1.5 ml. of 0.2 *M* phosphate buffer (pH 5.8), 0.5 ml. of the reduced cytochrome$_{551}$, 0.1 ml. of potassium cyanide, 0.1 ml. of cytochrome$_{GB}$, and 0.4 ml. of distilled water. (A), 0 *M*; (B), 2×10^{-4} *M*; (C), 4×10^{-4} *M* of potassium cyanide, in final concentration.

Like other cytochromes and blue protein, cytochrome$_{GB}$ can be easily reduced be DPNH-cytochrome c reductase, and yeast lactic dehydrogenase. In the reduced form, there is an absorption band around 550 mμ, while the broad absorption band of the oxidized form at 620–630 mμ increases slightly in intensity. Such enzymic reduction of cytochrome$_{GB}$ can occur only under anaerobic condition owing to its rapid autoxidizability, as shown in Fig. 7.

However, cytochrome$_{GB}$ catalyzes oxidations of reduced cytochrome$_{551}$, and reduced blue protein, but is scarcely active towards reduced cytochrome$_{554}$, as shown in Fig. 8. When cytochrome$_{GB}$ is supplemented with the DPNH-cytochrome c reductase system or the yeast lactic dehydrogenase system, electron transfer between DPNH or lactate and oxygen is possible, and the resulting oxygen uptake can be measured with a Warburg manometer (Fig. 9).

Oxidation of reduced cytochrome$_{551}$ by cytochrome$_{GB}$ proceeds most rapidly between pH 5.5 and 6.0, as shown in Fig. 10. And the oxidation is strongly inhibited by $2\times10^{-4}M$ potassium cyanide, as shown in Fig. 11.

Table I

Inhibition by Cyanide, and Cardon Monoxide of the Terminal Oxidation System of the Resting Cells, and of the Reconstructed, Water-Soluble, Terminal Electron-Transferring System

Oxygen consumption was measured in a Warburg manometer at 37°. Xo_2 represents the first ten minutes' reading. The reaction mixture contained 0.4 ml. of 0.2 *M* phosphate buffer, 1.0 ml. of resting cell suspension or cytochrome$_{GB}$, 0.2 ml. of 1 *M* sodium lactate, and distilled water to a total volume 2.0 ml. The center well contained 0.2 ml. of 20 per cent KOH. For resting cell suspensions, the buffer was pH 6.6, and for cytochrome$_{GB}$ solutions, the buffer was pH 5.8 and 0.1 ml. of yeast lactic dehydrogenase was added as electron donor. For experiments on carbon monoxide-inhibition, the gas phase was 10 per cent of oxygen and 90 per cent of carbon monoxide or nitrogen. Light irradiation was by a 60 w. tungsten lamp from 10cm.

Inhibitor		KCN (*M*)				CO (%)			
Final concentration		0	10^{-3}	0	10^{-3}	0	90	0	90
System		Resting cell		Soluble system		Resting cells		Soluble system	
Xo_2 ($\mu l.$)	In the dark	—	—	—	—	114	62	30	13
	In the light	25	6	26	12	108	51	27	18
Inhibition (%)		76		55		In the dark 46	In the light 53	In the dark 57	In the light 18

Cyanide inhibition is also observed when yeast lactic dehydrogenase system is used as the electron donor, as shown in Table I. Carbon monoxide inhibits the cytochrome oxidase activity of cytochrome$_{GB}$ in the dark and this inhibition is partially reversed by light, as shown in Table I. These phenomena seem to be similar to the inhibitions caused by cyanide and carbon monoxide on lactate oxidation in living cells of *Pseudomonas aeruginosa*, as shown in Table I.

The cytochrome oxidase activity of cytochrome$_{GB}$ shows specificity towards various hydrogen-donating, chemical reagents as shown in Table II, thus showing similarity to animal cytochrome c oxidase (*15*). However, its

specificity to other cytochromes is quite different from that of animal cytochrome c oxidase, as shown in Figs. 12 and 13. Cytochrome$_{GB}$ can oxidize animal and yeast ferrocytochrome c's slowly, while animal cytochrome c

TABLE II

Comparison of Specificity Between Pseudomonas Cytochrome$_{GB}$ and Bovine Cytochrome c Oxidase to Electron Donating Chemical Reagents

Oxygen consumption was measured by use of a Warburg manometer. Reactions with cytochrome$_{GB}$, were at 30° in 0.04 *M* phosphate buffer (pH 6.0), and reactions using cytochrome c oxidase, were at 25° in 0.01 *M* phosphate buffer (pH 7.0).

Substrate used 1×10^{-2} *M* final concentration	Oxygen consumed			
	Pseudomonas cytochrome$_{GB}$		Bovine cytochrome c oxidase*	
	µl.	%	*µl.*	%
p-Phenylenediamine	60	100	80	100
o-Phenylenediamine	30	50	5	6
m-Phenylenediamine	0	0	0	0
Hydroquinone	65	108	96	120
Pyrocatechol	22	37	25	31
Resorcinol	0	0	0	0
p-Aminophenol	81	135	41	51
o-Aminophenol	121	202	57	71
m-Aminophenol	0	0	0	0
Pyrogallol	86	143	69	86
Phloroglucinol	5	8	0	0
L-Ascorbic acid	144	240	—	—

* Figures from "Okunuki, K., *Acta Phytochim.*, **12**, 1 (1941)".

oxidase can not oxidize the reduced cytochrome$_{551}$, and the reduced blue protein at all.

Pseudomonas aeruginosa can form a nitrate-reducing system, especially when grown anaerobically in a medium containing nitrate. However, cytochrome$_{GB}$ could not be shown to have any nitrate or nitrite reducing activity.

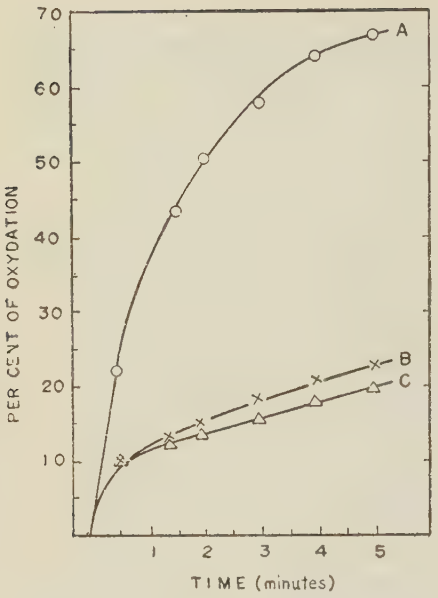


Fig. 12. Oxidations of the reduced *Pseudomonas* cytochrome$_{551}$, and reduced brkers's yeast-, and bovine heart muscle- cytochrom c's. Reaction mixture; 1.5 ml. of 0.2 *M* phosphate buffer (pH 5.8), 1.0 ml. of each of the reduced cytochromes, and 0.1 ml. of *Pseudomonas* cytochrome$_{GB}$. Extinction was measured at 551 mμ in the case of cytochrome$_{551}$, and at 550 mμ in the cases of the cytochrome c's. (A), P-cytochrome$_{551}$; (B), bovine cytochrome c; (C), yeast cytochrom c.

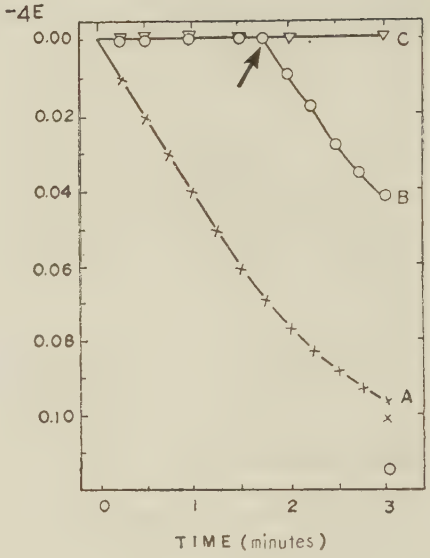


Fig. 13. Oxidations of reduced *Pseudomonas*- cytochrome$_{551}$, and blue protein, and reduced bovine cytochrome c by bovine cytochrome c oxidase. Reaction mixture; 1.0 ml. of 0.2 *M* phosphate buffer (pH 6.0), 1.0 ml. of each of the reduced respiratory components, 1.0 ml. of distilled water, and 0.5 ml. of bovine cytochrome c oxidase. (A), bovine cytochrome c; (B)*, P-cytochrome$_{551}$; (C), P-blue protein. The two isolated marks were measured after addition of a trace of ferricyanide. *At the time indicated by an arrow, a few drops of P-cytochrome$_{GB}$ was added to (B).

DISCUSSION

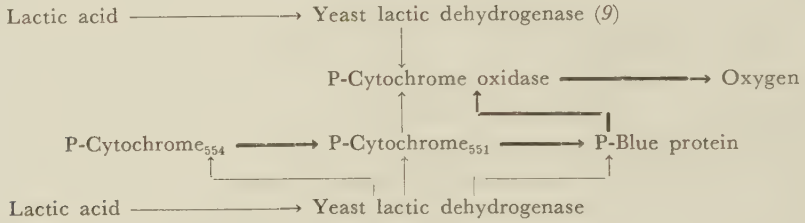
Of the cytochromes purified from *Pseudomonas aeruginosa*, cytochrome$_{551}$ is most similar to the cytochrome c's crystallized from baker's yeast and bovine heart muscle, in regard to the positions of its absorption maxima and its redox-potential. Examination of the absorption spectrum of cytochrome$_{551}$ shows that its α-band absorption maximum in reduced form is 0.5 to 1.0 mμ to the longer side of typical cytochrome c's. The redox-potential of the cytochrome from *Pseudomonas aeruginosa* are in the following order: P-blue protein > P-cytochrome$_{551}$ > bovine cytochrome c > P-cytochrome$_{554}$. Cytochrome$_{551}$ has a redox-potential nearest to that of bovine cytochrome c. In spite of such physical similarities, cytochrome$_{551}$ shows physiological properties distinct from those of the cytochrome c's: Cytochrome$_{GB}$ has cytochrome oxidase activity and can oxidize reduced cyto-

chrome$_{551}$, and reduced blue protein. However, it oxidizes reduced cytochrome c's very much slower than reduced cytochrome$_{551}$. On the contrary, cytochrome c oxidase from bovine heart muscle can oxidize reduced cytochrome c's but not reduced cytochrome$_{551}$, and reduced blue protein. This shows that reconstruction of terminal oxidation system should be made on materials prepared from one source. In the proceeding paper (*12*), it was shown that there is even a certain structural difference between yeast and bovine cytochrome c's. Kamen and Takeda (*3*) reported that a preparation of a cytochrome from *Pseudomonas* had an α-band absorption maximum at 552 mμ and they called it cytochrome c. In my work, the existence of such a cytochrome could not be shown, but instead, cytochrome$_{554}$, cytochrome$_{GB}$ and blue protein have been prepared from this same strain together with cytochrome$_{551}$. Blue protein has been purified into blue, needle-like crystals, and was found to contain copper. This protein is blue in oxidized form and when reduced, it loses its colour. This blue protein can be repeatedly oxidized and reduced physiologically in the presence of oxygen and cytochrome$_{GB}$, and of lactate and yeast lactic dehydrogenase, respectively in a similar way to the cytochromes. Therefore, it seems likely that other proteins than the so-called cytochromes may function as electron carriers in the terminal electron transferring system.

As shown in the previous report (*1*), the preparation of cytochrome$_{GB}$ shows absorption bands around 550 mμ and 620–630 mμ, which suggests that it might be contaminated with cytochrome$_{551}$, cytochrome$_{554}$ and blue protein. However, attempts to remove such pigments from the preparation of cytochrome$_{GB}$ were unsuccessful, suggesting that such absorption bands might be essential for the cytochrome oxidase activity of cytochrome$_{GB}$. The physiological studies described above have led to a conclusion that cytochrome oxidase activity of cytochrome$_{GB}$ is comparable with that of animal cytochrome c oxidase. Therefore, the preparation of cytochrome$_{GB}$ which was tentatively named after its greenish brown colour (*1*), should now be called *Pseudomonas* cytochrome oxidase. Fujita and Kodama (*16*) and Negelein and Gerischer (*17*) found spectroscopically that cytochrome a_2 was widely distributed among bacterial kingdom, was autoxidizable and could combine with carbon monoxide, and cyanide. These properties of cytochrome a_2 led to the assumption that it had the function of a cytochrome oxidase. Recently, Tissiéres (*18*) has demonstrated the cytochrome oxidase-like properties of cytochrome a_2 in the living cells of *Aerobacter aerogenes*. However it has also been suggested cytochrome a_1 might be the terminal respiratory enzyme of *Acetobacter pasteurianum* (*19*, *29*). But in neither case, is it known what substance is oxidized by these "oxidases". The living cells of *Pseudomonas aeruginosa* have in their reduced state spectroscopic absorption bands around 590 mμ and 630 mμ, corresponding to cytochrome a_1 and a_2, respectively. At present, P-cytochrome oxidase appears to be more similar to cytochrome a_2 than to cytochrome a_1, since it has an absorption maximum around 630 mμ. Only after further purification will it be

possible to decide whether P-cytochrome oxidase is similar to cytochrome a_1 or a_2.

From the above facts the following electron-transferring scheme is suggested:



In this scheme, several pathways are possible from lactate to oxygen. If TPNH- and DPNH- cytochrome c reductase from other sources are used in place of yeast lactic dehydrogenase, almost the same pathways are possible. However, it is probable that the reconstruction of the terminal oxidation system is confused if reducing systems prepared from other sources than *Pseudomonas aeruginosa* are used.

SUMMARY

Investigations were made on some physical and physiological properties of cytochrome$_{551}$, cytochrome$_{554}$, blue protein and cytochrome$_{GB}$, extracted and purified from a strain of *Pseudomonas aeruginosa*.

These four respiratory pigments can be electron-acceptors of yeast lactic dehydrogenase, and pig heart DPNH-cytochrome c reductase.

Redox-potentials of cytochrome$_{554}$, cytochrome$_{551}$ and blue protein were found to be +225 mV., +250 mV., and +300 mV., respectively. One of a lower potential can spontaneously transfer an electron to one of a higher potential. Cytochrome$_{551}$, and blue protein can donate electrons to cytochrome$_{GB}$, but not cytochrome$_{554}$, and cytochrome$_{GB}$ is oxidized by oxygen.

Some properties of cytochrome$_{GB}$ and bovine heart cytochrome c oxidase were compared. The results indicated that cytochrome$_{GB}$ has a cytochrome oxidase function in *Pseudomonas aeruginosa*.

Cytochrome$_{551}$ and blue protein were further purified, and the latter was crystallized into needle-like form.

The terminal electron-transferring system of *Pseudomonas aeruginora* was discussed on the basis of the results obtained.

I wish to thank Dr. T. Yamano for analysis of redox-potential and Dr. Y. Takeda for his advice. I should like to thank my colleagues, Messrs*. M. Nozaki, T. Yamanaka, J. Yamashita, and H. Mizushima, and Messrs.** K. Kusai and M. Nakai for helpful works and discussions during the course of this work.

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REFERENCES

(*1*) Horio, T., *J. Biochem.*, **45**, 195 (1958)
(*2*) Tissiéres, A., *Biochem. J.*, **64**, 582 (1956)
(*3*) Kamen, M. D., and Takeda, Y., *Biochem. et Biophys. Acta*, **21**, 518 (1956)
(*4*) Postage, J. R., *J. gen. Microbiol.*, **14**, 545 (1956)
(*5*) Ishimoto, M., Yagi, T., and Shiraki, M., *Symposia on Enzyme Chemistry* (*in Japanese*), **11**, 184 (1956)
(*6*) Mahler, H.R., Sarker, N.K., Vernon, L.P., and Alberty, R.A., *J. Biol. Chem.*, **199**, 585 (1952)
(*7*) Haas, E., Horecker, B. L., and Hogness, T. R., *J. Biol. Chem.*, **136**, 747 (1940)
(*8*) Haas, E., Harrer, C. J., and Hogness, T. R., *J. Biol. Chem.*, **143**, 341 (1942)
(*9*) Yamanaka, T., Horio, T., and Okunuki, K., *J. Biochem.*, in press
(*10*) Hagihara, B., Horio, T., Yamashita, J., Nozaki, M., and Okunuki, K. *Nature*, **178**, 629 (1956)
(*11*) Hagihara, B., Morikawa, I., Sekuzu, I., Horio, T., and Okunuki, K., *Nature*, **178**, 630 (1956)
(*12*) Nozaki, M., Yamanaka, T., Horio, T., and Okunuki, K., *J. Biochem.*, **44**, 453 (1957)
(*13*) Sekuzu, I., Takemori, S., and Okunuki, K., unpublished
(*14*) Smith, L., and Stotz, E., *J. Biol. Chem.*, **209**, 819 (1954)
(*15*) Okunuki, K., *Acta Phytochim.*, **12**, 1 (1941)
(*16*) Fujita, A., and Kodama, T., *Biochem. Z.*, **273**, 186 (1934)
(*17*) Negelein, E., and Gerischer, W., *Biochem. Z.*, **268**, 1 (1934)
(*18*) Tissiéres, A., *Biochem. J.*, **50**, 279 (1951)
(*19*) Smith, L., in "Methods in Enzymology" ed. by Colowick, S. P., and Kaplan, N. O., Acad. Press, N. Y., vol. **II**, p. 732 (1955)
(*20*) Chance, B., *J. Biol. Chem.*, **202**, 383 (1953)

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STUDIES ON PSYCHOSINE

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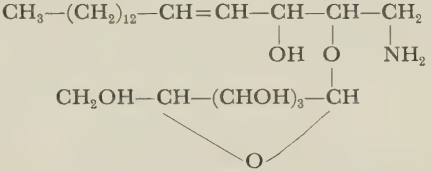
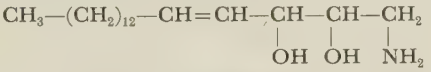
(Received for publication, December 23, 1957)

The author investigated previously a few methods for the isolation of sphingosine. Psychosine has been separated as a byproduct in that studies, and the attempt was made to study on this substance. For the first time, the psychosine was isolated form Kerasine by Thudichum (*1*) (1901), and later Klenk (*2*) (1926) also isolated by baryt method and presented the following constitutional formula (I).



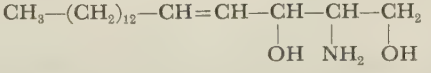
(I)

This formula was supported by the configuration of sphingosine (II), which was reported later by Klenk and Diebold (*3*) (1931).



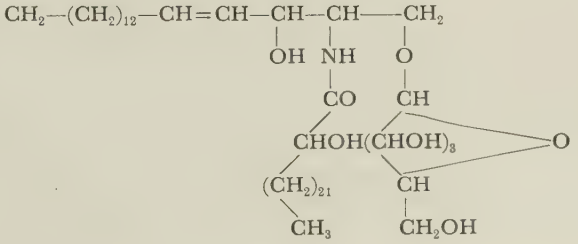
(II)

Recently Ohno (*4*) (1944) and Carter (*5*) (1947) had independently presented the same configuration of sphingosine,differing from that presented by Klenk. The configuration of sphingosine decided by them was as follows (III):



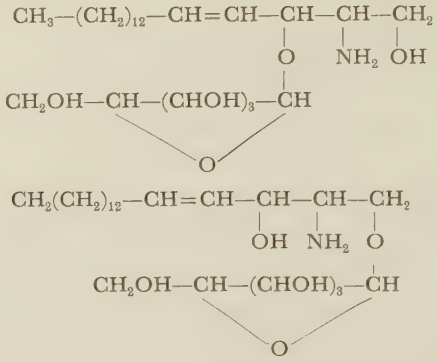
(III)

In regard to the position of the glycosidic linkage between sphingosine and galactose in cerebron molecule, Nakayama (*6*) (1950) reported the next formula (IV).



(IV)

Since the configuration of sphingosine was decided as described above, the glycosidic linkage between sphingosine and galactose in psychosine must be either (V), or (VI).



(V)

(VI)

If the configuration of psychosine is (VI), a C_2 unsaturated palmitic aldehyde (hexadecene-2-al) would be produced with lead tetraacetate oxidation after Criegee (*7*) (1937), but if it is (V), a higher fatty aldehyde would not be produced. Ohno has shown that hexadecene-2-al is produced with lead tetraacetate oxidation of sphingosine and it is extractable in *iso*-amylalcohol. The position of glycosidic linkage between sphingosine and galactose can therefore be decided by observing the product obtained by lead tetraacetate oxidation of psychosine.

Studies on this problem were carried out and the results show that the aldehyde produced with lead tetraacetate oxidation of psychosine is the same one as produced with oxidation of spyingosine.

EXPERIMENTAL

Psychosine isolated from beef brain by Klenk method (*8*) (1930) was used in this experiment. The analytical values were as follows:

	Sugar (%)	N (%)	Sphingosine (%)
Calcd.	39.0	3.04	64.9
Found	36.8	2.98	62.5

Oxidation of Psychosine with Lead Tetraacetate—2mg. of psychosine was put into a test tube, adding anhydrous benzene and lead tetraacetate, then was heated in the water bath at 75° for 10 minutes. After oxidation, a few drops of glycerol was added into tube and heated for a few minutes more. By this procedure excess lead tetraacetate was decomposed. After cooling to room temperature, water and petroleum ether were added and shaken vigorously. The layer of petroleum ether was transferred to another tube, then petroleum ether was evaporated to dryness under vacuum. The residue dissolved thoroughly in glacial acetic acid. When 10 ml. of Schiff's reagent was added to the solution, purple colour was developed. Therefore, after standing for 24 hours, 10 ml. of *iso*-amylalcohol was added to it and mixed thoroughly. A few minutes later the solution divided into two layers and it was found that the *iso*-amylalcohol layer showed violet colour.

So, tho colour was determined with photometer. The same procedure was repeated twice using different amount of psychosine.

The extinction obtained as follows:

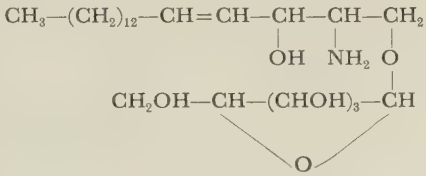
Amount of psychosin (*mg.*)	Sphingosine contained (*mg.*)	Volume of *iso*-amylalcohol (*ml.*)	Extinction
2.0	1.30	100	0.52
1.0	0.65	50	0.52
0.5	0.325	50	0,26

These results suggest that the structure of psychosine is (VI) and psychosine is quantitatively oxidised with lead tetraacetate under above described conditions.

SUMMARY

Studies were carried out on psychosine isolated from beef brain. It was shown that a higher fatty aldehyde which was identical with the one obtained from the lead tetraacetate oxidation of sphingosine was produced with lead tetraacetate oxidation of psychosine.

Based on this result it is thought that the glycosidic linkage between sphingosine and galactose exists in C_1 position of sphingosine and the structure of psychosine would be as follows:



The author wishes to express his deep indebtedness to Prof. K. Ohno for his helpful criticism on this work.

REFERENCES

(*1*) Thudichum, J.L.W., Die chemische Konstitution des Gehirns des Menschen und der Tiere, Franz Pietzcker, Tübingen (1901)
(*2*) Klenk, E., *Z. physiol. Chem.*, **153**, 74 (1926)
(*3*) Klenk, E., and Diebold, W., *Z. physiol. Chem.*, **198**, 25 (1931)
(*4*) Ohno, K., *J. Japanese Biochem. Soc.*, **18**, 151 (1944)
(*5*) Carter, H.E., *J. Biol. Chem.*, **170**, 285 (1947)
(*6*) Nakayama, T., *J. Biochem.*, **37**, 309 (1950)
(*7*) Criegee, R.Y., *Z. angew. Chem.*, **50**, 153 (1937)
(*8*) Thierfelder, H., and Klenk, E., Die Chemie der Cerebroside und Phosphophatide, Julius Springer, Berlin, p. 51 (1930)

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EFFECTS OF CARBOXYPEPTIDASES (ANSON'S AND BASIC) ON PROTAMINES*

Sirs:

During the course of studies on the chemical structure of protamines (*1—3*), the effects of a well-known carboxypeptidase (CPase) crystallized by Anson (*4*) and a basic carboxypeptidase (basic CPase) recently announced by Folk (*5*) upon the basic proteins have been studied in our laboratory, together with specificities of these enzymes towards substrates containing arginine in their C-terminal region. The results will be here briefly reported and later fully published elsewhere.

1. Action of DFP (Diisopropyl Fluorophosphate)-Nontreated CPase on Protamines.—Crystalline CPase, isolated from frozen bovine pancreas according to Anson's method (*4*) and recrystallized four times, was made to act upon

TABLE I

Action of DFP-Nontreated CPase on Protamines

Substrate	Digestion time (*hr.*)	0	2	5	24
Clupeine sulfate	Amino acid released[a]	—	Ala	Ala, (Ser, Val)[b]	Ala, Ser, Val
	Mole of amino-N increased/mole of clupeine sulfate[c]	—	—	0.82	0.91
Salmine sulfate	Amino acid released[a]	—	(Ser, Val)[b]	Ser, Val, Ala	Ser, Val, Ala
	Mole of amino-N increased/mole of salmine sulfate[c]	—	—	1.1_6	1.3_0
Iridine sulfate	Amino acid released[a]	—	(Ser, Val)[b]	Ser, Val, Ala	Ser, Val, Ala
	Mole of amino-N increased-mole of iridine sulfate[c]	—	—	1.1_4	3.0_0
Enzyme blank	Amino acid released[a]	—	—	—	—

* The contents of this paper were separately presented several times at Annual Meetings and Symposia of the Japanese Biochemical Society and the Chemical Society of Japan (27th Annual Meeting of the Japanese Biochemical Society, Sendai, April 1954; Symposium on the Protein Structure, Osaka, November 1954; Symposium on the Chemical Structure of Proteins, Tokyo, October 1957, and Joint Meeting of Chemical Societies in Japan, Tokyo, November 1957).

3.1μmole (20 mg.) of protamine sulfate and 0.12 mg. of enzyme protein N per ml. of reaction mixture at pH 7.6 adjusted with 0.1 *N* NaOH, 37°.

a) Identified by ninhydrin and Sakaguchi reactions on one-dimensional paper chromatogram, using acetic acid: butanol: $H_2O = 1:4:1$ (*v/v*) system.

b) Amino acids in parenthesis gave no obvious order of occurrence due to their similar coloration by ninhydrin.

c) Molecular weight of protamine sulfate was taken for 6,500.

protamines. Aliquots pipetted from the digestion mixture were boiled to stop the enzymic reaction, and the amino acids released in the solution were identified by one-dimensional paper chromatography. An increase in the α-amino groups in each solution was also determined by Van Slyke's manometric method. The results were summarized in Table I.

TABLE II

Action of DFP-Treated CPase on Protamines

Experiment No.	Substrate	Molar ratio[a] of substrate: enzyme	Temp.	pH, Buffer	Amino acid liberated during 0–48 *hr.*
1	Clupeine sulfate	225, 100 and 20:1	37°	7.8, adjusted with *N* NaOH	None
2		75:1	37°	7.6, *M*/15 Phosphate	None
3		13 and 1:1	25°	7.6, 0.6–0.8*N* LiCl–0.05*M* veronal	None
4		100:1	37°	7.6, adjusted with *N* NaOH	None
5	Salmine sulfate	50:1	37°	7.8, adjusted with *N* NaOH	None
6	Iridine sulfate	50:1	37°	7.8, adjusted with *N* NaOH	None

In experiment No. 3, six times recrystallized CPase according to Neurath (*9*) was used. Twelve times recrystallized CPase according to Anson's method (*4*) was used in all other experiments. In exp. No. 4, a specimen of the enzyme was used after treatment with DFP, followed by dialysis against distilled water.

In experiments Nos. 2 and 3, the molecular sieve method of Partridge (*6*) was applied to the hydrolysate.

As developing solvent for paper chromatography, formic acid : butanol : $H_2O = 15:75:10$ (*v/v*) system was used, and liberated amino acids were colored with ninhydrin and Sakaguchi reactions.

a) Molecular weight of protamine sulfate and CPase, respectively, was taken for 6,500 and 34,300 (*9*).

2. Action of DFP-Treated CPase on Protamines—As shown in Table II, DFP-treated CPase in this case was made to act upon protamines under

various conditions. Liberated amino acids were investigated on one-dimensional paper chromatogram either by the same method as in the former case or by using the molecular sieve method of Partridge (*6*) treating with Amberlite IR-120 (H form). From the result it has been found that the CPase has essentially no action upon the protamines. It has also been shown that the protamines never inhibit the action of CPase on its substrate, carbobenzoxy-glycyl-L-phenylalanine (CGP).

3. Action of CPase on N-Acyl Dipeptides Containing Arginine in N- or C-Terminus—Substrates used in this experiment were synthesized by utilizing the mixed anhydride method (*7*, *8*). In this case, a specimen of CPase

TABLE III

Action of CPase on N-Acyl Dipeptides Containing Arginine in N- or C- Terminus

N-Acyl dipeptide containing C-terminal arginine	Hydrolysis[a] % (0–48 *hr.*)	*N*-Acyl dipeptide containing N-terminal arginine	Hydrolysis % (20 *min.*)
Cbz*-Gly-L-Arg	0	Bz*-L-Arg-Gly[b]	15.4
Cbz-D, L-Ala-L-Arg	0	Bz-L-Arg-L-Leu[c]	42.5
Cbz-L-Leu-L-Arg	0	Bz-L-Arg-L-Phe[c]	62.0
Cbz-L-Phe-L-Arg	0	Bz-L-Arg-L-Glu[b]	20.6
Cbz-L-Pro-L-Arg	0		

0.02 *M* Substrate concentration, 0.05 *M* veronal buffer containing 0.2 *N* NaCl, pH 7.5 at 25°. Hydrolysis per cent was determined by ninhydrin colorimetry. Liberated amino acids were identified as described in Table II.

a) Enzyme concentration, 1.43×10^{-2} mg. of protein N/ml. of reaction mixture.

b) Enzyme concentration, 1.01×10^{-2} mg. of protein N/ml. of reaction mixture.

c) 10 per cent (*v*/*v*) methanol was added to the buffer. Enzyme concentration, 2.02×10^{-3} mg. of protein N/ml. of reaction mixture.

* Cbz=Carbobenzoxy; Bz=Benzoyl.

was used which was isolated by Neurath's procedure (*9*), recrystallized 7–9 times (proteolytic coefficient 18 at an initial concentration of 0.02 *M* CGP) and treated with DFP just prior to the use. As clearly seen from the results indicated in Table III, such a purified CPase has shown none of the action upon substrates containing C-terminal arginine, but on the contrary, easy liberation of each C-terminal residue from substrates containing arginine just adjacent to the C-terminal.

4. Action of Basic CPase Fraction on Protamines—Changes in the basic CPase activity towards carbobenzoxy-glycyl-L-arginine of some fractions obtained were examined during the course of isolation and purification of

CPase by the method of Neurath (*9*). It has been shown, that the activity decreased in the order of the supernatant from once crystallized CPase (SPN), the baryta extract between pH 4.6—6.0 from euglobulin precipitate (BE) and once crystallized CPase (CPXO), whereas the CPase activity increased in that order. Furthermore, it is in accord with the result of Folk (*5*), that the basic CPase activity was shown to be practically delected from CPase by repeated recrystallization.

In the present experiment, the SPN fraction, in which the strongest activity of basic CPase was demonstrated, was made to act upon clupeine and salmine, after the treatment of the fraction with DFP, followed by dialysis against distilled water overnight. Identification of the amino acids released was made by means of one-dimensional paper chromatography after treatment with molecular sieve (*6*), and measurement of increase in the α-amino groups by ninhydrin colorimetry of each digestion mixture. These results are indicated in Fig. 1. Of these resultant digestion mixtures, of

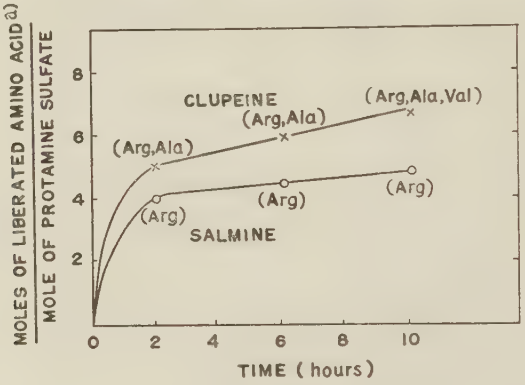

Fig. 1. Action of basic CPase fraction (SPN, in text) on protamines.

0.5 μmole (3.25 mg.) of protamine sulfate and 0.022 mg. of enzyme protein N per ml. of reaction mixture, with 0.05 *M* veronal buffer containing 0.1 *N* NaCl, pH 7.5 at 25°. Identification of amino acid in parenthesis, see the footnote (*a*) of Table I.

a) Moles of the ninhydrin α-amino groups liberated per mole of protamine sulfate (molecular weight taken for 6,500 as sulfate).

two hours, each of clupeine and salmine was separated into monoamino acids, arginine and protamine core, using a column of Amberlite IRC-50 (XE-64) buffered at pH 8.0 with 0.1 *M* borate buffer containing 0.6 *M* sodium chloride*. Monoamino acids and arginine were determined by ninhydrin and Sakaguchi methods, respectively (Table IV). The protamine cores were dinitrophenylated under the condition of Levy (*10*), and the *R*

* This method of separation was kindly suggested by Dr. Ishii in this laboratory and will be fully reported by him elsewhere.

TABLE IV

Analysis of Two Hour Basic CPase-Digestion Mixture of Protamines

Substrate	Monoamino acid liberated, mole[a)]	Arginine liberated, mole[a)]	*R* Value of DNP-protamine core
Clupeine sulfate	0.19	3.70	0.74 (0.75)[b)]
Salmine sulfate	0.00	3.80	0.89 (0.92)[b)]

a) Moles of amino acid liberated per mole of protamine sulfate (molecular weight taken for 6,500 as sulfate), monoamino acid determined by ninhydrin and arginine by Sakaguchi reaction.

b) Parenthesized value shows the value of DNP-protamine for control.

values (the ratio of optical densities at 390/360 mμ of absorption curve) (*2*) of the DNP-derivatives were observed. As also shown in Table IV, the *R* value of each DNP-protamine core was in good agreement with that of each DNP-protamine for control, indicating presumably that little change has occurred concerning tne N-terminus. Thus the enzyme fraction, when treated with DFP, is considered to have caused no cleavage at internal peptide linkages but have released amino acids stepwise from the C-terminus of the protamine.

Moreover, the action of DFP-treated BE and CPXO fractions, obtained from another preparation, upon iridine* and salmine, respectively, has shown qualitatively similar results as in the above.

In conclusion, it has been shown that, Anson's CPase (CPase A) does not split C-terminal arginine in peptides and proteins, while "basic" CPase (CPase B) surely splits. As the result, clupeine and salmine have been confirmed to have arginine in their C-termini**, and also have presumably an average of 3–4 residues of arginine collectively in that region. Thus it has been demonstrated, that the CPase B serves as a useful tool in the C-terminal structure study of proteins, getting over the barrier of basic amino acids to the action of CPase A.

REFERENCES

(*1*) Ando, T., Ishii, S., Yamasaki, M., Iwai, K., Hashimoto, C., and Sawada, F., *J. Biochem.*, **44**, 275 (1957)

(*2*) Ando, T., Abukumagawa, E., Nagai, Y., and Yamasaki, M., *J. Biochem.*, **44**, 191 (1957)

(*3*) Ando, T., Nagai, Y., and Fujioka, H., *J., Biochem.*, **44**, 778 (1957)

* Carried out by Dr. Hashimoto. The result will soon be published.

** The same result has been obtained by hydrazinolysis experiment made by Dr. Kawanishi in this laboratory. Details will be reported later.

(*4*) Anson, M. L., *J. Gen. Physiol.*, **20**, 663 (1937)
(*5*) Folk, J. E., *J. Am. Chem. Soc.*, **78**, 3541 (1956)
(*6*) Partridge, S. M., *Nature*, **169**, 493 (1952)
(*7*) Van Orden, H. O., and Smith, E. L., *J. Biol. Chem.*, **208**, 751 (1954)
(*8*) Hofmann, K., Peckham, W. D., and Rheiner, A., *J. Am. Chem. Soc.*, **78**, 238 (1956)
(*9*) Neurath, H., Methods in Enzymology, edited by Colowick, S. P. and Kaplan, N. O., Acad. Press, New York, Vol. **II**, p. 77 (1955)
(*10*) Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L., Methods of Biochemical Analysis, edited by Glick, D., Interscience Publ., New York, Vol. **II**, p. 359 (1955)

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